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**Biodegradation of phthalate esters using
efficient microorganisms**

DHARMENDRA KUMAR



DIVISION OF MICROBIOLOGY
ICAR- INDIAN AGRICULTURAL RESEARCH INSTITUTE,
NEW DELHI-110012

2019

दक्ष सूक्ष्मजीवों द्वारा थैलेट एस्टर्स का जैव अवक्रमण
**Biodegradation of phthalate esters using efficient
microorganisms**

By

DHARMENDRA KUMAR

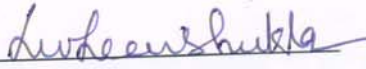
A Thesis

Submitted to the Post-Graduate School,
ICAR- Indian Agricultural Research Institute, New Delhi,
in partial fulfilment of requirements
for the award of degree of

**DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY
2019**

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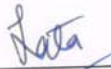
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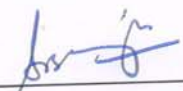
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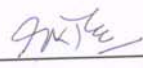
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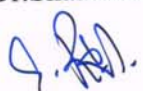
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Dr. Livleen Shukla
Principal Scientist

CERTIFICATE

This is to certify that the thesis entitled “**Biodegradation of phthalate esters using efficient microorganisms**” submitted to the Faculty of the Post-Graduate School, **ICAR-Indian Agricultural Research Institute, New Delhi**, in partial fulfilment of **Doctor of Philosophy in Microbiology**, embodies the results of bona fide research work carried out by **Mr. Dharmendra Kumar**, Roll No. 10311 under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help availed during the course of investigation as well as source of information have been duly acknowledged.

Place: New Delhi

Date: 5.02.2019

(Dr. Livleen Shukla)

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Advisory committee

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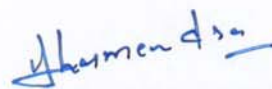
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(DHARMENDRA KUMAR)

Dedicated to my parents...
I am blessed to be your son



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1. INTRODUCTION

In recent years the worldwide increase of urbanization and industrialization has led to the release of some complex and toxic organic compounds into the environment. The presence of these contaminants called “emerging” contaminants (ECs) such as phthalates has been reported in many countries.

Phthalate esters (PAEs) are a group of compounds, which include dialkyl or alkyl aryl esters of 1,2-benzendicarboxylic acid (Phthalic acid). Phthalate esters or phthalic acid esters (PAEs) have acquired huge attention from environmental regulatory bodies owing to its endocrine disrupting anti-androgenic activity. Results of recent research have led to defining several PAEs as “environmental hormones” because they interrupt biological hormones (Kambia *et al.*, 2001 and Ahuactzin-Perez *et al.*, 2018) to cause great impact on living creatures. The long-term accumulation of PAEs in human may interfere with the secretion of hormones and the normal function of the nerve and immune systems, causing significant damage.

Among PAEs, dimethyl phthalate (DMP), dimethyl terephthalate (DMT), diethyl phthalate (DEP) and dibutyl phthalate (DBP) are mostly used for a variety of commercial purposes and have been listed as priority pollutants by agencies such as U.S. Environmental Protection Agency. Phthalate esters also have been widely used to increase flexibility, extensibility, and workability of plastic polymers as plasticizer or to help make perfume fixatives, lubricants, adhesives, weather stripping, and safety glass. Many consumer products contain specific members of this family of chemicals, including building materials, household furnishings, clothing, cosmetics, pharmaceuticals, nutritional supplements, medical devices, dentures, children's toys, glow sticks, modeling clay, food packaging, automobiles, lubricants, waxes, cleaning materials, and insecticides. Phthalates are synthetic compounds used predominantly as additives in plastic to improve mechanical properties of the plastic resin, particularly flexibility and absence of plasticizer renders a plastic brittle or inflexible. (Ren *et al.*; 2018 and Zhu *et al.*; 2018).

Low-molecular-weight phthalates (C3–C6) are widely used as additives in personal care products, viz. perfumes, lotions, cosmetics, toys, adhesives, inks, varnishes whereas high-molecular-weight phthalates (C7–C11) are primarily used as plasticizers in polyvinyl chloride-based plastic products. They are also used in

consumer products including flooring and wall coverings, paper coating materials, lubricants, cellulosic and styrene materials to increase their flexibility and durability (Zhang *et al*; 2018).

Due to their wide use, PAEs are found in human residential and occupational environments in reasonable concentrations due to their application in both industrial processes and consumer products. Various plastic materials are widely used in agriculture which includes soil fumigation film, irrigation drip tape, tubing, nursery pots, and silage bags. Since phthalates are not chemically bound to the plastics but remain present as a freely mobile and leachable phase, their migration from soft plastic during the production phase or via leaching or volatilization from plastic products in due course of their use in the environment and after disposal. So it is expected that soil may contain a high concentration of these PAEs which may find their way into the food chain.

Removal and degradation of PAEs by abiotic processes such as hydrolysis and photo decomposition has been reported to be slow and insignificant. However, breakdown by microorganisms is considered to be one of the major routes of environmental degradation for these widespread pollutants. Number of investigators has demonstrated successful degradation of several PAEs by microbes under aerobic condition in soil, natural water and wastewater. Although there are number of reports on the biodegradation of individual PAEs, there is scanty literature available on degradation of mixture of PAEs. Industrial wastes frequently contain mixture of PAEs and therefore it is important to investigate the biodegradation of their mixtures rather than individual PAEs (Zhu *et al*; 2018).

Several studies on the distribution and contamination level of PAEs in sediments have been reported worldwide, which reveal that polluted sediments are adversely affecting the ecosystem (Zeng *et al*; 2008). However, there is only one report available on the extent of levels of contamination in sediment samples of Gomti River, India (Srivastava *et al*; 2010). Therefore, it is necessary to assess the levels of these contaminants under different agricultural practices in order to devise strategies to bioremediate PAEs if present in high concentration.

Biodegradation of phthalate esters involves sequential degradation of the ester linkage by ester hydrolysis first forming the monoester with one carboxylic group and subsequently phthalic acid (PA) with two carboxylic functional groups

(Gu *et al*; 2004). Biodegradation is considered to be one of the major channels of metabolic breakdown of PAEs due to their low rate of chemical hydrolysis and photolysis alone. It is the key mechanism for microorganisms to degrade PAEs in the environment. During the degradation of PAEs, oxygenase and esterase play the key role. Firstly esterase specifically acts upon the ester bonds, resulting in the formation of respective monoester and alcohol or phthalic acid (PA). During the hydrolytic process dioctyl phthalate, butyl octyl phthalate, DBP, diethyl phthalate(DEP), monomethyl phthalate (MMP) and 1,3-isobenzofurandione are identified as few intermediate products of PAEs. It means that long alkyl side-chains are removed by oxidation and then the ester groups are broken by hydrolase (Wu *et al*; 2010 and Xu *et al*; 2017).

Successful degradation of PAEs and other phthalate esters by several microorganisms (bacteria, fungi, and algae) carried out effectively by ester hydrolysis with the help of different hydrolytic enzymes viz., esterases, peroxidases and oxygenases respectively. These hydrolytic enzymes play key role in their degradation and many Enterobacteriaceae members are known to degrade persistent xenobiotics.

Several studies have been reported worldwide in recent years on biodegradation of different phthalate esters by bacteria, fungi, algae and activated sludge culture (Liang *et al*; 2008 and Ren *et al*; 2018).

However, all these studies were performed in laboratories and the success of these microorganisms *in-situ* is still a big question. The goal of present investigation was to demonstrate the biodegradation of individual phthalate esters or their mixture in aqueous phase and soil using a newly isolated efficient bacterial strain alone or in combination.

Objectives:

1. Isolation and identification of Phthalate esters utilizing microbes from contaminated sites.
2. To study the degradation of Di-ethyl phthalate (DEP) and Di-2-ethylhexyl phthalate (DEHP) by isolated microbes.
3. *In-situ* Phthalate esters degradation potential of microorganisms under microcosm conditions.

2. REVIEW OF LITERATURE

2.1. About PAEs

Phthalate esters (PAEs) belong to a broad group of compounds which include Dialkyl or alkyl aryl esters of 1,2-benzene, Dicarboxylic acid (phthalic acid). PAEs are bis (2-Ethylhexyl phthalate (DEHP), Dibutyl phthalate (DBP), Diethyl phthalate (DEP) and Dimethyl phthalate (DMP) are regularly used for different commercial purposes (Figure 1)

Although, USA Environmental Protection Agency (EPA) has categorized PAEs as priority pollutants. But currently, PAEs are used as plasticizers due to performance cost, durability, and overall product sustainability benefits. As per the use, PAEs are the widest class of plastic additive. PAEs are produced by reacting phthalic anhydride with appropriate alcohol (generally 4 to 13 carbons) (Aguilar-Alvarado *et al*; 2015, Alexnder *et al*; 1994). They appear as colorless, odorless liquids. On the basis of solubility, PAEs have low water and high octanol to water partition coefficient and low volatility. With increasing alkyl chain length, hydrophobicity gets increased since the log Kow increases. The worldwide production and/or usage of phthalate and DEHP mentionrd in the Table 2.1.

PAEs have wide applications in various fields mainly industrial, agricultural and domestic child cares products but the most important application is their use as nonreactive plasticizers for improving the flexibility and workability of polymeric materials. PAEs increase the plastic flexibility when added to plastic as the long polyvinyl molecules slide against one another. PAEs have been detected at relatively high levels in soil, sediments, and waters as they are mainly discharged from the manufacturing processes into the environment. (Staples *et al.*, 1997; Tan *et al*; 2007; Roslev *et al*; 2007, 1998; Zou *et al*; 2002; Li *et al*; 2005, 2012; Chen *et al.*, 2011 and Kong *et al.*, 2012)

The production of PAEs first started during the early 1920s and is being produced in high amount since the mid-twenty century. These are more than 70 kinds of PAEs being produced in the present days. The production of PAEs exceeds 10 million tons in the world annually and more than 20 billion pounds of PAEs are used primarily as plasticizers. They are being used to increase the flexibility in different

polyvinyl chloride (PVCs) products as well as an inert ingredient in many sprays including herbicide, insecticide, pesticides and in many consumer products such as wood finishes and cosmetics. (Xie *et al.*, 2007, 2011, Lu *et al.*, 2009 and Boon *et al.*; 2003).

As the majority of the products are being used by a human being so they are regularly liable to PAEs through food, medicines, plastics, dairy products, and cosmetics, etc. PAEs and their metabolites which are produced during its degradation time have been reported to cause much reproductive and developmental disorder.

It has been reported that PAEs are causing estrogenic, hepatotoxic, teratogenic, carcinogenic and endocrine disruptive effects *in vivo* PAEs can easily transfer from plastic materials to the environment. They are generally found in soil, water air, food products, animal and the human body. Most PAEs: DEP, DBP, and DEHP have been found in the environment. They are primarily released during use and migrate from the packaging to contaminate the contents such as food and beverages. (Saeger *et al.*; 1976; Chen *et al.*; 2017; Van Wezel *et al.*, 2000; Prokupkova *et al.*, 2002; Hens and Caballos; 2003; Feng *et al.*, 2005; Holadova *et al.*, 2007; Matsumoto *et al.*; 2008; Cai *et al.*, 2008; and Xia *et al.*, 2011)

Many studies have been carried out where the presence of PAEs has been established in various environmental media such as water, air, solid and sediments and They also have been found in epoxidized soybean oil, soft spreadable cheese, sauces, peanut butter and different types of vegetables release of PAEs by abiotic processes such as hydrolysis and photodecomposition has been reported to be slow and insignificant. However, bioremediation is considered to be one of the major routes of environmental degradation for these widespread pollutants. Many of investigators have demonstrated successful degradation of several PAEs by microbes under aerobic conditions in soil, solids, sediments, water and wastewater (Ma *et al.*, 2003; Xie *et al.*, 2007; Pedersen *et al.*, 2008; Cai *et al.*, 2008; Zeng *et al.*, 2008; Lu *et al.*; 2009; Srivastava *et al.*, 2010; Wang *et al.*, 2012; Shaikh *et al.*; 2012; Sharma *et al.*; 2001 and Kong *et al.*, 2012).

2.2. Physical and Chemical Properties of PAEs

PAEs are characterized by low water solubility and high octanol/water partition coefficient, the physical and chemical properties define the fate of chemicals

Table 2.1: Worldwide production and/or usage of phthalates and DEHP

Countries	year	Productions	References
USA	2006	DEHP: 45 000-230 000 tons /year	USEPA 1991
European union	1997	1000000 tons /year	Lin <i>et al.</i> 2009
China	2007	DEHP: 305 000e340 000 tons	USEPA 1991
Canada	1991	DEHP: 10 000 tons PVC: 49%	Minister of Supply and Services, Canada, 1994
Germany	1997	240 000 tons/year	Kurane, 1997
Japan	2004	340 000 tons/year	Kurane, 1997
Sweden	1989	DEHP: 2.4 kg/person,Population: 8 400 000.	Nakamiya <i>et al.</i> 2005 Ejlertsson <i>et al.</i> 1996

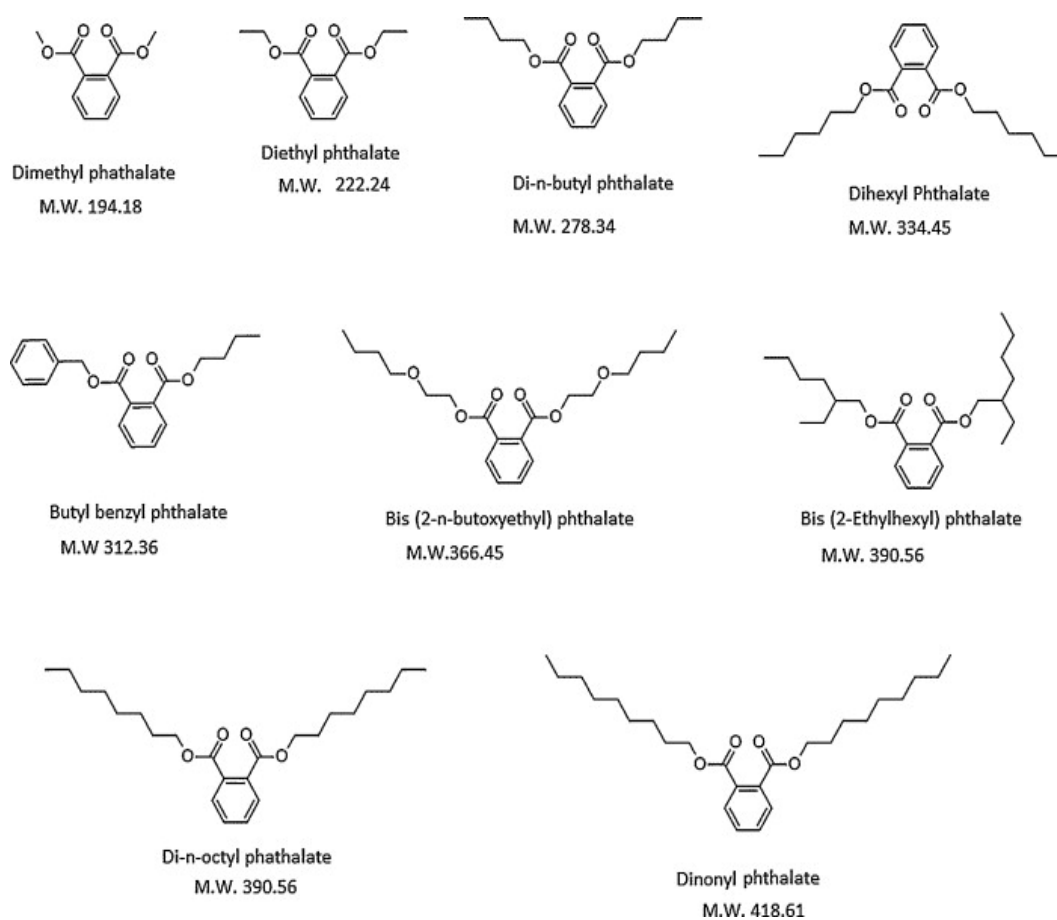


Figure1: Structure of few PAEs present in soil as contaminants

in the environmental compartment. The physical and chemical properties of some common phthalates are given in Table 2.2. PAEs can be categorized according to their molecular weight. PAEs with higher molecular weight, such as DEHP, DiNP, and DiDP, are primarily used as plasticizers to soften polyvinyl chloride (PVC) products, whereas PAEs with lower-molecular-weight (ester side chain length, one to four carbons), such as DMP, diethyl phthalate (DEP), din-butyl phthalate (DBP), and butyl benzyl phthalate (BBP), are widely used as solvents to hold color and scent in various consumer and personal care products (NRC, 2008). The physical and chemical properties of PAEs showing in Table 2.2.

2.3. Uses of various type of PAEs

According to USEPA, PAEs like DEHP, DOP, DBP, and DMP have been placed as a group of priority environmental pollutants and are become the most widely distributed classes of organic contaminants which have attracted much attention on a while global level (Kong *et al.*, 2012 and USEPA, 2013).

PAEs are extensively used as additives in the manufacture of plastics, paint, and cosmetics to increase their flexibility, transparency, durability, and longevity. Among all the uses PAEs mainly used in the manufacturing of PVC while DBP is used in epoxy resins while DMP and DEP are typically used in cellulose ester-based plastics (Feng *et al.*; 2017 and Cai *et al.*, 2003).

The use of PAEs as mostly plastic additives due to their excellent properties and compatibility with PVCs. They represent more than 90% of the Western European plasticizers market. DEHP accounts for about 50% of the total production of PAEs whereas DEP and DBP are another high volume commodity PAEs. It has been seen that the PAEs are part of the composition of soft PVCs, carpet backings, coatings, paints, glues, cosmetics, plastic medical devices, and other quotidian elements.

Many studies have shown that agricultural soils and protected vegetable cultivation have been contaminated by toxic pollutants. Some researchers have found that toxic pollutants such as heavy metals, Polycyclic aromatic hydrocarbons(PAHs), persistent organic pollutants and PAEs are present in agricultural soils (Gao *et al.*, 2005; Tao *et al.*, 2005; Hu and Ding, 2009; Chen *et al.*, 2011; Xia *et al.*, 2011 and Kong *et al.*, 2012).

The use of DMPs is widespread as they are used in cellulose ester-based plastics, paints, adhesives, printing inks, and coatings, etc. owing to its extensive applications in industries; it has been categorized as a priority environmental contaminant. It has been found that it promotes chromosomal injuries in human leucocytes, also known to be an endocrine-disrupting chemical that interferes with the reproductive system and normal development of animals and human beings. It is a stable compound in the natural environment with a half-life of about 25 years. The use of DMPs in cellulose ester-based plastic such as cellulose acetate and butyrate is quite common and it is a component of coating food packing, cosmetics, lubricants, decorative clothes, etc. (Tang *et al.*; 2016 and Staples *et al.*, 1997).

The other common PAEs, DEP is mostly used as a solvent vehicle for fragrance as well as in cellulose ester-based plastics. It has been also reported not only to cause abnormality in sexual differentiation but it also acts as an endocrine-disrupting chemical. The use of DBP is mostly found in cosmetics, children's toys and childcare products. It has been reported to reduce human sperm production and its motility, decrease rates of pregnancy and also promote miscarriages (Murature *et al.*;1987; Fredricson *et al.*;1993; Gray *et al.*; 2000 and Chang *et al.*; 2007).

DEHP is a high production volume chemical used in the manufacture of a wide variety of consumer food packaging, some children's products, and some PVC and medical devices. Reports have shown that the respective mean concentrations of DBP and DEHP, especially in greenhouse soils, were 1.63 and 1.96 mg /kg in Jinan, Shandong province (Meng *et al.*, 1996; Ma *et al.*, 2003; Xu *et al.*, 2008; Crinnion *et al.*; 2010 and Chen *et al.*, 2011).

The amount of Diethyl Phthalate and bis (2-Ethylhexyl phthalate) in plastic film greenhouse and polytunnel soils are remarkably influenced by differences in the use of irrigation and Plastic film mainly comprises the walls of polytunnels or for mulching at the soil surface in intensive protected vegetable cultivation, fertilizers and plastic film as well as meteorological conditions and external pollutant sources PAEs can also lead to atmospheric or water pollution by evaporation, leaching, deposition and drainage in soil (Xu *et al.*, 2008; Mo *et al.*, 2008; Cai *et al.*, 2008; Zeng *et al.*, 2008; Kong *et al.*, 2012 and Wang *et al.*, 2016).

Table 2.2: Physical and chemical properties of PAEs (Cao *et al*; 2010)

PAEs	Weight	Density (gmL ⁻¹)	Vapor Pressure 25 °C (Pa)	Water Solubility (mgL ⁻¹) at 25 °C	LogKow at 25 °C
Dimethyl phthalate (DMP)	194.2	1.191	0.263	5220	1.61
Diethyl phthalate (DEP)	222.2	1.232	6.48×10^{-2}	591	2.54
Dipropylphthalate (DPP)	250.3	1.078	1.75×10^{-2}	77	3.4
Di-iso-buthyl phthalate (DiBP)	278.3	1.039	4.73×10^{-3}	9.9	4.27
Di-n-buthyl phthalate (DiBP)	278.3	1.043	4.73×10^{-3}	9.9	4.27
Buthylbenzyl phthalate (BBzP)	312.4	1.119	2.49×10^{-3}	3.8	4.70
Di-n-hexyl phthalate (DHP)	334.5	1.011	3.45×10^{-4}	0.159	6.00
Di-2-ethyl hexyl phthalate	390.6	0.985	2.52×10^{-5}	0.285	7.73
Di-n-octyl phthalate (DOP)	390.6	0.985	2.52×10^{-5}	2.49×10^{-3}	7.73
Di-iso-nonyl phthalate (DiNP)	419	0.972	6.81×10^{-6}	3.08×10^{-4}	8.60
Di-iso-decyl phthalate(DiDP)	446.66	0.966	1.84×10^{-6}	3.81×10^{-5}	9.46

Greenhouse cultivation has expanded dramatically in China since the 1980s, reaching up to 3.5 million ha by 2011 (Li *et al.*, 2012). Greenhouse cultivation is mainly for vegetable production in China, and plastic greenhouses account for more than 99% of greenhouse cultivation relative to glass greenhouses (Costa *et al.*, 2004). Several studies detected PAEs in soils of vegetable greenhouses in Nanjing and Hangzhou, as well as in other agricultural soils, such as vegetable soils in Guangzhou and paddy soils in Leizhou Peninsula in China (Wang *et al.*, 2013; Chen *et al.*, 2011, Cai *et al.*, 2005; Khai *et al.*, 2007 and Guan *et al.*, 2007). The buildup of PAEs in agricultural soils may contaminate agricultural products, and further raise the human health risk (Guan *et al.*, 2007). The Shandong Peninsula is the largest Peninsula in China with rapid urbanization and a high population density of 550 people/km². The Peninsula includes the cities of Qingdao, Yantai, Weifang, and Weihai. The Shandong Peninsula has a long history of vegetable greenhouse cultivation and is a main vegetable-producing region, with its greenhouse coverage accounting for approximately 50% of that of China. The vegetable greenhouses in this peninsula are close to the highly populated urban areas, and plastic film is widely used. The plastic film of 30000 tons/yr is estimated to be used only in one county, i.e., Shouguang in Weifang of Shandong Peninsula. PAEs account for 10 wt% to 60 wt% of plastic products, thus giving rise to concerns about the potential risk of PAEs in recent years. However, few studies focused on the characteristics of PAEs in the soils of vegetable greenhouses in the Shandong Peninsula. This study provides information on the concentrations, compositions, and distributions of 16 PAEs in soils from vegetable greenhouses in the Shandong Peninsula and discusses possible sources, influence factors, and potential environmental risk (Plate 1).

2.4. Role of PAEs in the contamination of our environment

The USEPA has decided admissible or maximum concentration in water of 6 mg/l for DEHP. The threshold limit value of DEHP, DBP and DMP are 0.55, 0.45, 5.0 and 5.0 mg/l respectively. There are threshold level of DEHP, DEP and DMP decided for different types of water like in case of drinking water in the range is 0.02–0.6 g/l, for 0.1–100 g/l, for surface water 0.2–110 g/l for treated wastewater and 0.1–12g/l for treated industrial wastewater (USA EPA 1991).

It has been observed that PAEs can enter into the environment during the manufacturing stage, use and disposal of plastics. It is well that PAEs and their

metabolites are suspected endocrine-disrupting chemicals exhibiting carcinogenic action. Nowadays, as the farming system is advancing so is the use of large amounts of plastic-like films, pipes in agriculture and bags in fertilizers which increase the chances of pollutants of PAEs. As farmers are using plastic equipment in intensive vegetable which might increase the health problems due to PAEs through food consumption. As result high quantity of PAEs in agricultural soils have already been reported in the Pearl River Delta (McKee *et al.*, 2004; Cai *et al.*, 2005; Xu *et al.*, 2008; Mo *et al.*, 2008; Pedersen *et al.*, 2008; Zhao *et al.*, 1998; Zeng *et al.*, 2001, 2008; Gu *et al.*, 2011; Zhang *et al.*, 2016; and Kong *et al.*, 2012).

It is a known fact that soil is considered an environmental matrix which can migrate pollutants to plants and finally enter the human food chain. DBP has a bad impact on the ascorbic acid and capsaicin contents of chili vegetables. The uptake of DEHP by nine types of greenhouse vegetable cultivation varied from 10.14 to 36.16 mg/ kg. As per the investigation carried out in the last three decade investigations on the occurrence and degradation of PAEs it has been found to be increasing in various soils. The six PAEs which have been mentioned earlier have been widely identified in the soils from different regions in China. (Yin *et al.*, 2003; Peijnenburg and Struijs; 2006; Cai *et al.*, 2008; Zang *et al.*, 2008; Fu and Du; 2011 and Wen *et al.*; 2015).

The studies were carried out in different cultivated fields such as upland, having plastics film greenhouse, kitchen gardens, vegetable fields, orchard fields, cotton fields, and crop fields as well as paddy fields. It was observed that the various cultivated types and land usages induced different distribution patterns of PAEs in soils. As there is maximum usage of PVC plastics films containing PAEs as well as fertilizers containing PAEs so the higher PAEs concentration was reported in protected greenhouse and vegetable soils. Further, it was found that Vegetable and orchard fields showed higher PAEs concentrations than rice and cotton fields since vegetable and orchard fields generally require intensively pesticide and fertilizer use, and plastic packing. Another report also indicated that the study carried out with poultry manure and commercial fertilizers where plastic usage maximum DBP and DEHP accounted for about 68.1-99.4% of the PAEs. It has been further concluded that the higher is the concentration of PAEs in soil. It was also observed as the time passes the PAEs components in plastic films are discharged to soils and the more the soils

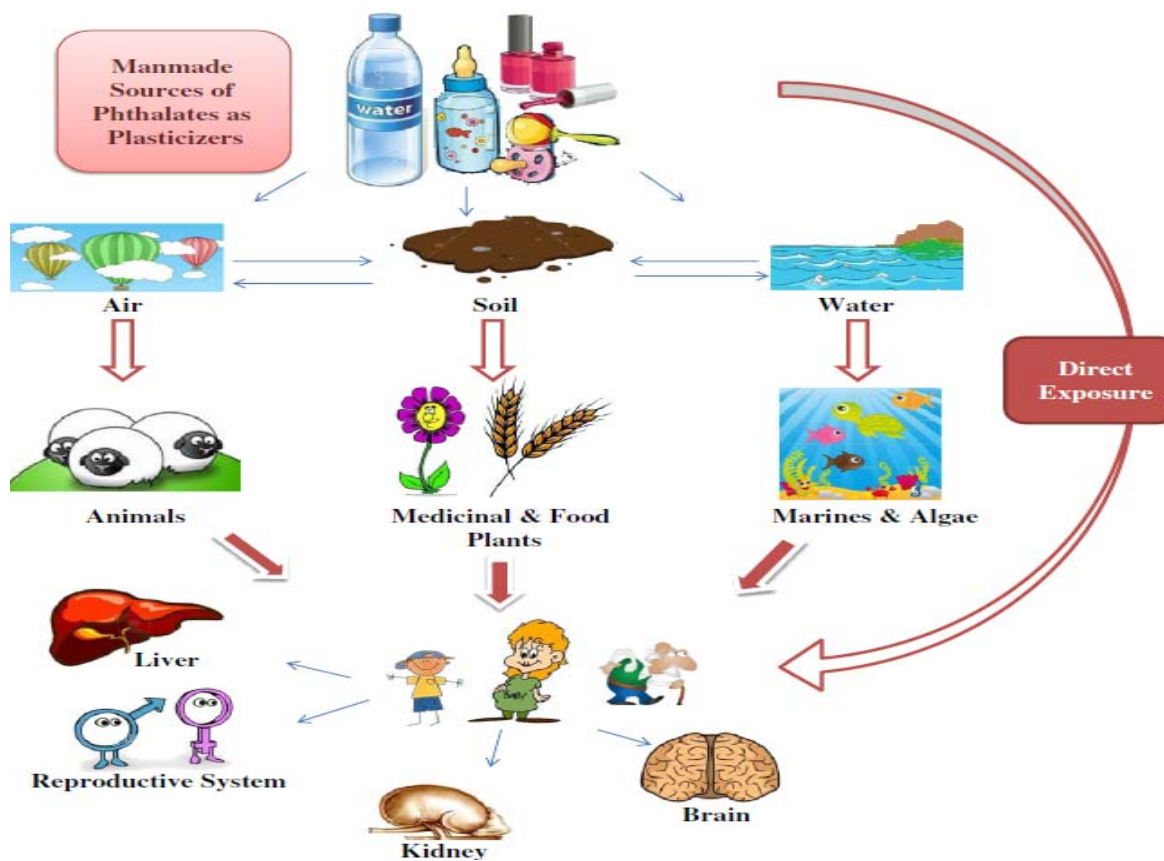


Plate 1: Entry of phthalate esters through different routes into food chain

are covered with plastic film *i.e.* duration, the PAEs concentration in the soil also becomes high. It has been observed that the PAEs' concentration in the soil also becomes high. It has been observed that the PAEs might be present in pesticides, irrigation water, and aerial deposition are also potential sources and require investigation in future studies (Chou *et al*; 2006; Van Wezel *et al.*, 2000; Hens and Caballos; 2003; Batlle *et al*; 2004; Xu *et al.*, 2008; Zeng *et al.*, 2008 and Kong *et al.*, 2012).

The major human exposure sources for phthalates include sources such as material related (building material, furniture, electronic devices), product-related (cosmetics, packaging, paints, textiles, and medical devices), industrial (production, manufacturing, exhaust air), agricultural (insecticides, pesticides and drugs), and wastes (landfills and waste sludge). DEHP, which is the most recalcitrant PAE compound, is found in a variety of products including from dialysis tubing, paints, adhesives, as well as in food products because of leaching during production or storage. Castle *et al*;1990 measured DEHP levels in milk samples collected from a dairy in Norway in which plasticized tubing was used as milking equipment.

The reported concentration of DEHP in milk samples collected from the milking chamber ranged from 30 µg/kg to 50 µg/kg, on the other hand, it was 5 µg/kg in control samples obtained by hand milking. Studies suggested that daily exposures of infants and children to DEHP from the ingestion of breast milk, cow's milk, and infant formula are in the range of 1–10 µg/kg/d (Zhou *et al*; 2006). Exposure to DEHP in patients with chronic renal failure undergoing maintenance hemodialysis was investigated by Dine *et al*; (2000) and the leached amount of DEHP from the plastic dialysis tubing was measured during a 4-h dialysis session.

They reported that the concentration of DEHP in blood plasma measured at the outlet of the dialysis machine (before the patient) found to be increased from 0.4 to 2.6 µg/ml during a 4 h dialysis session. DEHP is also found in a variety of food products because of leaching during production and storage. DEHP, DBP, BBP, and DEP were detected in both the packaging and the contacted foods Levels of DEHP was low (0.065 µg/g on average in beverages and 0–29 µg/g on average in foods) associated with the use of DEHP-plasticized cap or lid seals (Cao *et al*; 2010).

DEHP, DBP, BBP, and DEHP were found in butter and margarine that was covered with aluminum foil and paper laminates. Migrated DEP from pie cartons was

measured as 1.8 µg/g (average) (Page and Lacroix, 1995). PAEs belong to the class of endocrine-disrupting pollutants and they show reproductive and developmental health effects on biological organisms. They can reduce concentrations of testosterone, an important androgen (or male sex hormone) that contributes to the development of male sex organs. Laboratory studies on the determination of the developmental effect of phthalates on rats suggested that di-n-hexyl phthalate and dicyclohexyl phthalate are responsible for a reduction in fetal weight, a decrease in the anogenital distance in a female fetus, and undescended testis in the male fetus. Reddy *et al*; (2006) suggested that there is an association between the occurrence of endometriosis disease and concentrations of PAEs in the plasma of women suffering from endometriosis. Researchers reported that the correlation between the concentrations of PEs and different severity of endometriosis was strong and statistically significant for di-butyl phthalate (DnBP), butyl benzyl phthalate(BBP), di-n-octyl phthalate (DNOP), and diethyl hexyl phthalate (DEHP). Swan and Davis (2003) also reported that DEHP leads to changed serum cholesterol levels, decreased serum estradiol levels, prolonged estrous cycles in rats, and no ovulation in adult cycling rats. In addition to the health effects of PAEs on the endocrine system, some PAEs also have carcinogenic health effects on animals. For example, DEHP was shown to produce cancer in rodents after high-level lifetime exposures (Tickner *et al*; 2001 and Ikonomou *et al*; 2008).

However, studies that investigated the mechanism of peroxisome proliferation suggested that animal studies are not relevant for humans since humans are much less sensitive than rodents to PPAR-alpha mediated effects.

According to USEPA's carcinogenicity weight-of-evidence classification, DEHP was classified as B2, probable human carcinogen based on increased liver tumors in adult male and female rats. In addition, the diethyl phthalate was classified as class D which means not classifiable as to carcinogenicity (USAEPA, IRIS, 2013).

Carcinogenicity classification of PAEs:

Compound	Carcinogenity Class
DEP	Group C
DEHP	Group C
DMP	Group D
DBP	Group B2

B2: probable human carcinogen, C: possible human carcinogen
D: not classifiable as a human carcinogen

2.5. Impact of PAEs on soil and human health

PAEs are present in daily consumable things such as it has been investigated that they have estrogenic character and their high environmental diffusion has been the reason for which regulation on water and air concentrations, disposal and worker exposure criteria have been formulated, mainly for the most heavily used PAEs (Howdeshell *et al*; 2008).

The various investigation agencies of Europe and America, namely the USA Agency for Toxic Substances and Disease Registry (USA EPA NTP-CERHR, TSDR, 1995, 1997, 2001, 2002) the Centre for the Evaluation of Risks to Human Reproduction (NTP CERHR, 2000, 2003 and 2005), USA EPA, European Chemicals Bureau (ECB, 2004), European Food Safety Authority (EFSA, 2004), European Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE 1998, 2004) and International Agency on Research on Cancer (Nakajima, IARC, 2009) are engaged in the study and analysis of risk assessments on PAEs.

Studies on the effect of PAEs were carried out on rodents and it was found that PAEs are known to show low value for acute toxicity with LD 50 values of 1 to 30 g/kg bodyweight. A few short and long term dose-related studies in rodents have also shown adverse effects in liver, kidney, thyroid gland tissue and testes for the experimental animals and quite significant differences could be reported not only in different species as well as between males and females (Wang *et al*; 2017; Ding *et al*; 2015 and Dine *et al*; 2000).

PAEs were found to be endocrine disruptors that interface with the body's endocrine system and produce adverse reproductive, developmental, neurological and immune effects in both humans and wildlife. When PAEs tested for mutagenicity and genotoxicity, they showed a negative effect for the same and DBP was also found to be associated with tumors promoting activity. The exposure to DEHP produced hepatocellular carcinoma in rodents along with a variety of other hepatocellular effects such as the proliferation of peroxysomes and mitochondria increase in Cyp4A1 and PCoA activities, liver tissue proliferation, suppression of apoptosis, etc (NTP-CERHR, 2000). It was also observed that most of these effects are mediated by induction of the PPAR-alpha receptor whereas when administered to mice DEHP does not result in hep to cellular effects. Later it was observed that various reasons like differences in PPAR alpha density, regulation, and signaling pathways, etc. adverse

effects associated with PPAR activation in rodents do not occur in humans (NTP-CERHR, 2002, 2003, 2005). A recent re-evaluation of DEHP by IARC the classification from “possible carcinogenic to humans” has been changed to “not classifiable as to carcinogenicity in humans” (Nakajima, IARC, 2009).

There is a rise in toxicological concern regarding the possible endocrine-disrupting potency of phthalates. The potential of phthalates to cause adverse effects on reproduction and development in humans was evaluated by the National Toxicology Program Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR, 2005).

Due to many reasons *e.g.* differences in PPAR alpha density, regulation and signaling pathways, adverse effects associated with PPAR activation in rodents do not occur in humans (NTP-CERHR, 2005). So IARC recently re-evaluated DEHP and changed its classification from “possible carcinogenic to humans” to “not classifiable as to carcinogenicity in humans” (Nakajima, IARC, 2009).

Recently toxicological concerns rise regarding the possible endocrine-disrupting potency of phthalates. The potential of phthalates to cause adverse effects on reproduction and development in humans was evaluated by the National Toxicology Program Centre for the Evaluation of Risks to Human Reproduction (NTP-CERHR, 2005).

Due to the maximum exposure to humans is due to bio magnifications since they are at the top of food chains. PAEs are easily released during the production, distribution, waste disposal and can easily be leached out from landfills into the water, soil and groundwater and as per recent concern, PAEs are ubiquitously present in the environment and have been described as manmade environmental priority pollutants

2.6. Techniques for quantification of PAEs

The most common technique for PAEs estimation is gas chromatography accompanied with detection through electron capture or in some cases mass spectrometry. Further, it was found that in order to simplify sample treatment prior to GC analysis of PAEs solid-phase microextraction has been widely used in the last decade. Various techniques like Spectrophotometry, gas chromatography, high-performance liquid chromatography, and fluorescent spectrometry have been utilized for the determination of PAEs. Although GC is mostly preferred for being simple,

rapid and sensitive in comparison with HPLC GC also has some drawbacks like high column and gasification temperatures (Castillo *et al*; 1998; Kato *et al*; 2006; Guo *et al*; 2010, 2011; Gangula *et al*; 2010 and Cacho *et al*; 2012).

On the other hand, HPLC is useful for the analysis of organic compounds especially isomeric mixtures and metabolites. HPLC is also superior to GC when it is used for the estimation of compounds with high boiling points. Mass spectrometry is also an important detection technique for PAEs as it has high sensitivity, specificity and excludes interference from impurities. A series of GC–MS and HPLC–MS methods have been reported for the estimation of PAEs in different materials. As the PAEs have complicated sample matrix and low concentration levels so the direct estimation of PAEs is not possible (Gibson *et al*; 1984, 2005; Liao *et al*; 2010 and Net *et al*; 2016)

The most important criterion for PAEs analysis is accuracy and efficiency which is very difficult because of the non-availability of a clean reagent blank. If the levels of PAEs in the reagent blanks show a lot of variation, it is difficult to report a figure as the result.

Hence, to solve this problem the two main steps separation and pre-concentration steps are required before the estimation. In order to monitor PAEs, many pre-concentration techniques like liquid-liquid extraction, solid-phase extraction, solid-phase microextraction and cloud point extraction are being used extensively. All these techniques have their merit and demerit hence their application for the estimation of PAEs according to different situations (Cacho *et al*; 2012).

It has been confirmed that to determine PAEs at sub mg/l levels, a pre-concentration step is the utmost necessary before analysis. Even for the estimation of PAEs in water samples, various extraction methods have been used such as solid-phase extraction, solid-phase microextraction, and dispersive liquid-liquid microextraction have gained importance for estimation of PAEs in water samples. Liquid-liquid extraction is one of the simple methods for the extraction of PAEs but this method needs high amount of sample as well as organic solvents. Now the liquid-liquid extraction method has been modified and it can be performed using a small amount of solvent (Nelson *et al*; 1996, 2008; Prokupkova *et al*., 2002; Feng *et al*., 2005; Farahani *et al*., 2007; Holadova *et al*., 2007; Liang *et al*., 2008; Luo *et al*; 2012; and Gou *et al*; 2011).

2.7. Bioremediation of PAEs

Many methods including adsorption, hydrolysis, TiO₂ photocatalysis pulse radiolysis, and electron beam radiolysis, photodegradation, phytoremediation, and microbial degradation are few methods which are helpful for the removal of PAEs from the environment. The two approaches use for bioremediation that is bioaugmentation and biostimulation. Three methods used for bioremediation is mineralization, biotransformation, and cometabolism (Wu *et al.*, 2012; Gu *et al.*, 2004; Yuan *et al.*, 2002; Lu *et al.*, 2009; Li *et al.*, 2006, Chen *et al.*, 2015, 2017 and Chao *et al.*, 2004, 2006). (Figure 2)

2.7.1. Phytoremediation

There is very little information regarding the phytoremediation of PAEs in soils. The different aspects of phytoremediation like phytovolatilization, phytostabilization, phytodegradation, phytoextraction, and phytoaccumulation, etc.

All the soil having high levels of contamination especially with persistent organic pollutants needs phytoremediation as a final clean up step. Among the microorganisms, rhizospheric and rhizoplane microorganisms play the most important role in the process of phytoremediation and attention has increased the importance of the metabolic activity of the soil microbial community in contaminated soils. Legumes crops like alfalfa and soybean have been used to eliminate organic pollutants from contaminated soils. *Sedum plumbizincicola* and *Elsholtzia splendens* are two major plant species that have been widely used in soil polluted with hazardous toxic inorganic elements and have shown a good ability to extract copper and zinc from contaminated soils. (Figure 3)

2.7.2. Microbial remediation

Microorganisms as a remedial resource have great versatility and they are simple, environment-friendly and less expensive when compared to other nonbiological techniques for contaminating treatment. Bioremediation involves the degradation of pollutants with the help of the enzymes present in living organisms. Microorganisms involved in bioremediation belong to the aerobic, anaerobic or facultative group of microorganisms. (Xu *et al.*, 2007; Liang *et al.*, 2008; Lu *et al.*, 2009; Wu *et al.*, 2010, Chang *et al.*, 2002, Xu *et al.*, 2005, Benjamin *et al.*, 2016, Chang *et al.*, 2004, Chatterjee and Dutta, 2003; Chao *et al.*, 2006 and Kumar *et al.*, 2017).

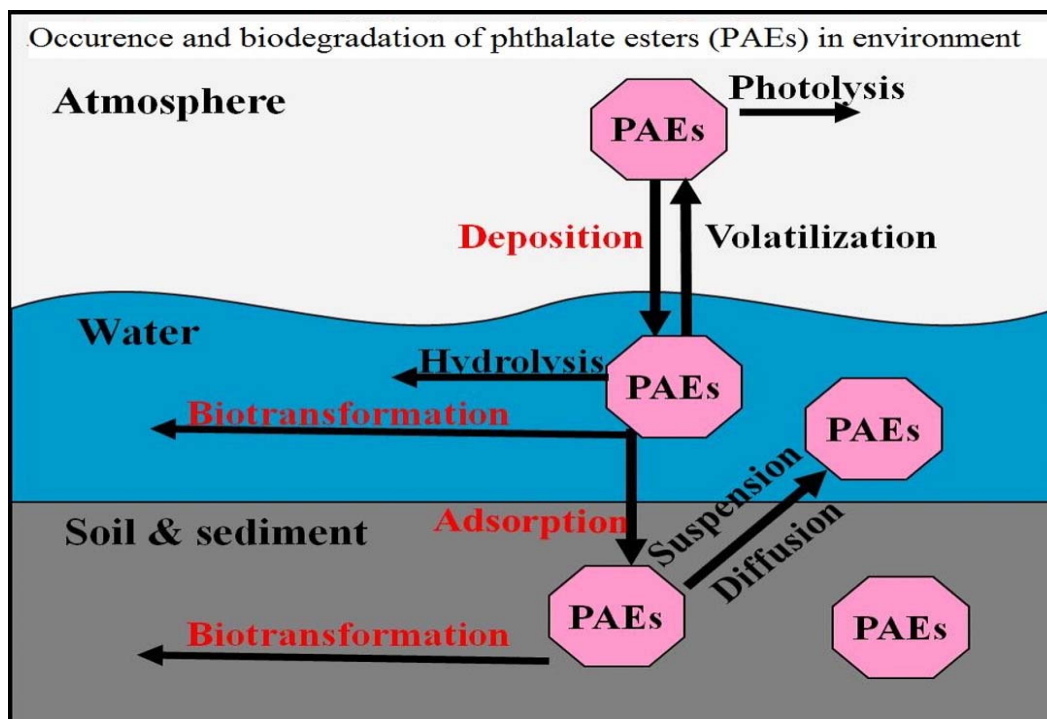


Figure 2: Various methods involved during degradation of phthalate esters (PAEs)

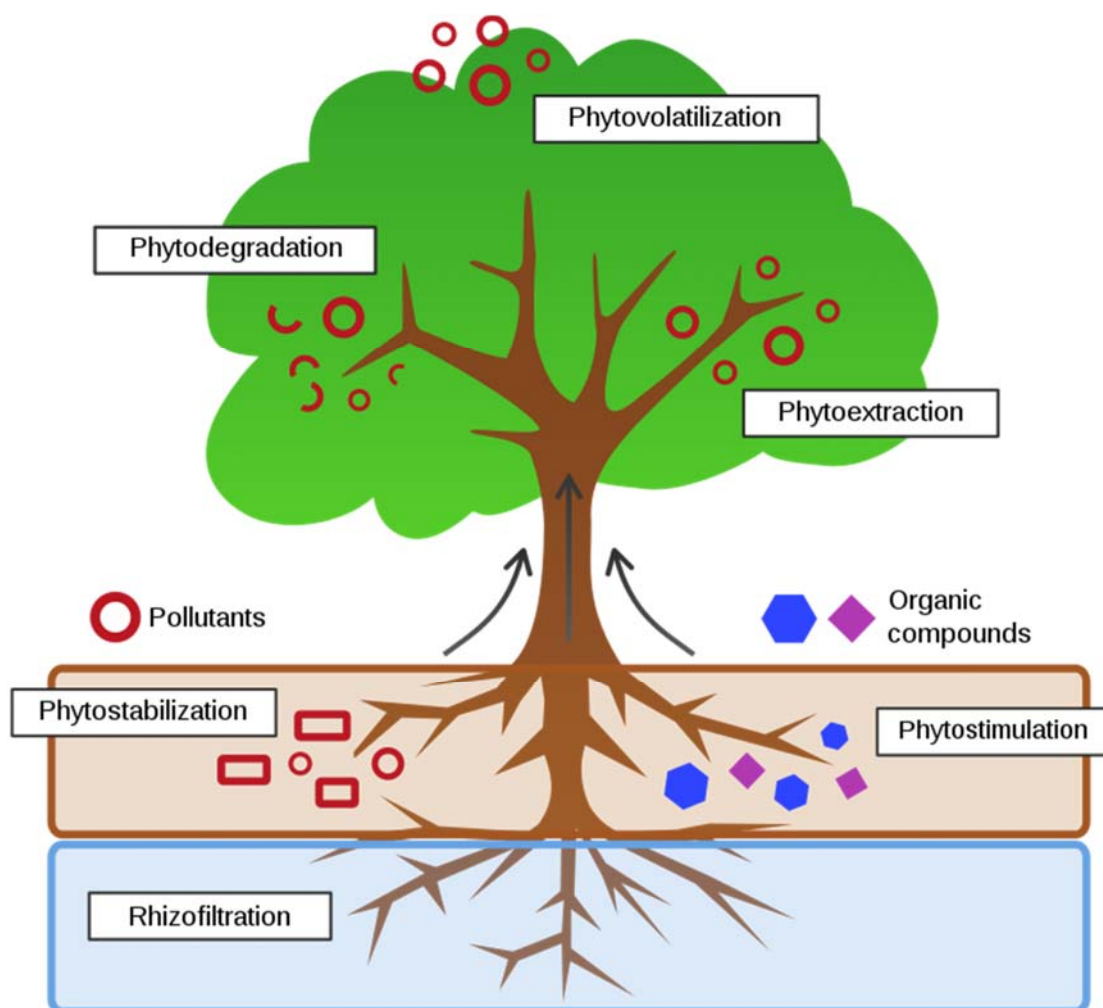


Figure 3: Phytoremediation of phthalate esters in nature

Many bacterial species have been isolated from activated sludge, mangrove sediment, soil, and solids, wastewater, river sludge, etc. with the ability to degrade PAEs among different bacterial strains included are from about 25 genera such as *Pseudomonas*, *Sphingomonas*, *Achromobacter*, *Rhodococcus*, *Enterococcus*, *Gordonia*, *Cornebacterium*, and *Agrobacterium*. (Wu *et al.*, 2010; Nakamiya *et al.*; 2005; Karegoudar *et al.*, 1984; Wang *et al.*, 1995, 2000; Shelton *et al.*, 1984; Gao *et al.*; 2016 and Ren *et al.*; 2017, 2018).

2.7.3. Mechanisms for degradation of PAEs

Studies carried out for the phthalate structure showed that they consist of the benzene ring and dicarboxylic acids having two side chains. The side chains can be alkyl. It has also been shown that various phthalates like DEP, DMP, DBP, and BBP consist of short ester chains and they are easily degraded as well as mineralized. On the other side, PAEs with longer ester chains such as DEHP, DOP, and DHP are resistant to degradation. The difference in biodegradability of phthalates has been found to be due to the presence of side chains ester chains causing steric effect due to which hydrolytic changes hindered resulting in non binding and inhibition to hydrolysis. (Chen *et al.*, 2000, 2004; Ahuactzin-Perez *et al.*; 2018 and Zhang *et al.*; 2018)

2.7.4. Application of pure culture

The most common genera found in all four divisions of bacteria. Bacteria having the capability for PAEs degradation are categorized into four divisions:

1. Proteobacteria
2. Actinobacteria
3. Firmicutes
4. Bacteroids

Actinobacteria have high G+C% while Firmicutes have low G+C%. Aerobic or facultative anaerobes microorganisms are capable of degrading PAEs. Among all the four divisions of bacteria, the most commonly found genera are *Pseudomonas*, *Arthrobacter*, *Comamonas* as well as *Sphingomonas* and *Rhodobacter*. (Chang *et al.*; 2004 and Wang *et al.*; 2000). The microbes community observation also noticed that community change was accompanied by the genera *Sphingomonas*. It has been reported

that different PAEs isomers show different rates of biodegradation and that the PAEs hydrolyzing enzymes are structurally specific for their action (Ejlertsson *et al*; 1995).

In addition to bacteria, a few fungal species including *Phanerochaete chrysosporium*, *Penicillium lilacinum*, *Sclerotium rolfsii*, *Aspergillus niger* AG-1, *Chlorella pyrenoidosa*, *Trametes versicolor*, *Fusarium*, *Daldinia concentrica*, and *Polyporus brumalis* have been reported to degrade PAEs. (Engelhardt *et al*; 1975, Yan *et al*; 2002; Lee *et al*; 2004; Pradeep *et al*; 2015 and Wang *et al*; 2017). Fungi show a wide range of degradation ability which is mainly due to the very strong extracellular ligninolytic enzymes such as manganese-dependent peroxidase, lignin peroxidase, and laccase. The best illustration is the cutinase producing *Fusarium oxysporum* which reported high enzymatic activities and was able to degrade PAEs (Kim *et al*. 2002, Kim *et al*; 2003, 2005; Patil *et al*; 2006; Xu *et al*; 2017; Li *et al*; 2015, 2016; Kumar *et al*; 2017; and Ahn *et al*; 2006) within several days.

2.7.5. Application of microbial consortium

In nature mostly different microorganisms syntrophically carry out the complete biodegradation or mineralization of complex organics, like PAEs. The bioremediation of PAEs primarily involves the sequential hydrolysis of ester linkage due to this there is the formation of monoesters and alcohols simultaneously (Gu *et al*; 2005 and Ren *et al*; 2016, 2018).

Hence, it has been observed that the microbial assimilation of PAEs requires diverse metabolic genes and enzymes which directly showed that a single organism is likely able to completely mineralize PAEs (Staples *et al*; 1997).

It was reported that during the biodegradation of DMP isomers by *Rhodococcus ruberosa* corresponding intermediates mainly monomethyl phthalate isomers were not further degraded (Li *et al*; 2005, 2010 and Wang *et al*; 2015).

It was also reported that dibutyl phthalate metabolites monobutyl phthalate and mono benzyl phthalate could not be further mineralized by the DBP degrading bacteria *Gordonia* sp. MTCC 4818 (Chatterjee and Dutta 2003) while Engelhardt and Wallnofer (1978) further investigated that a dibutyl phthalate degrading bacterium could not biodegrade mono benzyl phthalate.

So far it has been observed that instead of single microorganisms, the mixed culture consortia have shown to mineralize phthalates completely. Wang *et al*. (2004)

reported that the complete mineralization of DMP was observed with two consortia of microorganisms; one comprised of *Pseudomonas dihydroxynas fluorescens*, *Pseudomonas aureofaciens* and *Sphingomonas paucimobilis*, whereas the other comprised of *Xanthomonas maltophilia* and *S. paucimobilis* respectively.

When a co-culture consisting of an *Arthrobacter* sp. and *Sphingomonas paucimobilis* were reported for the degradation of DMP, it was found that these two microorganisms were able to degrade DMP serially. *Arthrobacter* sp was able to transform DMP into MMP which was utilized by *S. paucimobilis*.

It was further confirmed that BBP was also completely mineralized by a consortium consisting of *Arthrobacter* sp. WY and *Acinetobacter* sp. FW. It was investigated that *Arthrobacter* sp.WY was able to metabolize BBP into MbuP, MBzP, and Phthalic acid respectively while *Acinetobacter* sp. FW was capable of degrading deesterified alcohol resulting in the complete BBP mineralization (Chatterjee and Dutta 2008) Studies have shown that there is need of co-culturing is also required for the degradation of phthalates under anaerobic condition and it was seen when *Methanospirillum hungatei*, *P.terephthalaicum* JTT, and *P.isophthalicum* JTT were used as co-culture, there was complete degradation of TA and IA respectively (Qiu *et al*;2006).

In addition, mixed cultures have reported an increase in PAEs' degradation rates. As it was reported that when both *Sphingomonas* sp. DK4 and *Corynebacterium* sp. O18 were used for degradation, the degradation rates of all the 8 phthalates examined were enhanced (Chang *et al*; 2004 and Pradeep *et al*; 2015).

2.7.6. Pathways for biodegradation of Phthalates

The whole mechanisms of PAEs biodegradation guided to know the mineralization as well as the production of toxic metabolite if any. The main step for the biodegradation of phthalates consists of the conversion of PDFs to PMEs. PMEs are further degraded to phthalic acid and finally CO₂ released or CH₄ is released. (Nozawa and Maruyama 1988; Staples *et al*; 1997, Zhao *et al*; 2016 and Horn *et al*; 2004).

2.7.7.1. Primary pathway

There is a different type of pathways for degradation of PAEs but pathways involved in degradation are β -oxidation, trans-esterification, de-esterification, and dealkylation.

β -oxidation: is followed when PAEs with longer side chains than Diethyl Phthalates are generally transformed to those with shorter chains and it involves the removal of one ethyl group each time. After that Diethyl Phthalate is further converted to Phthalic acid by two pathways by de-esterification and an alternative transesterification. (Amir *et al*; 2005).

De-esterification: is the most common pathway where PDEs are converted to PMEs and Phthalic acids frequently. This biodegradation pathway has been studied for both aerobic and anaerobic conditions and has been found to be similar under both conditions and the study was performed with atleast 25 bacterial genera (Eaton and Ribbons 1982, Shelton *et al*; 1984 and Yan *et al*; 2015).

Trans-esterification/demethylation: Diethyl Phthalate can be easily biodegraded by changing the ethyl group ($-C_2H_5$) with a methyl ($-CH_3$) group in each step and results in the production of ethyl and Dimethyl phthalate and this whole process is known as trans-esterification (Cartwright *et al*; 2000). *Arthrobacter sp.* is able to biodegradation of PME into Phthalic Acid, the method direct hydrolysis of DMP. While *Aureobacterium saperae* NRRL B-14840 degrade Diethyl phthalate esters to Phthalic Acid. (Jackson *et al*; 1996).

Degradation pathway: During the biodegradation of PAEs and PAHs including phenanthrene the most common or a central intermediate is Phthalic Acid. It has been observed that under aerobic and anaerobic conditions the pathways of ring cleavage of Phthalic Acid are different (Cartwright 2000 and Eaton *et al*; 2001).

It has been found that under the aerobic condition the biodegradation of Phthalic Acid is involved, dioxygenase induced catabolic pathway where protocatechuate is the common intermediate. Gram-negative bacteria first uses a dioxygenase to form, cis-4,5- dihydro-4,5-dihydroxyphthalate as catalysis which is further oxidized to 4,5-dihydroxyphthalate using NAD-dependent dehydrogenase and further decarboxylation of 4,5-dihydroxyphthalate using decarboxylases result in the formation of protocatechuate cleaved while in case of carried out by two dioxygenase catalyzed pathways forming the common intermediate protocatechuate. (Eaton and Ribbons 1982, Yan *et al*; 2015 and Nomura *et al*; 1992).

Among the bacteria studied it has been reported that *Pseudomonas fluorescens* and *Pseudomonas putida* use the ortho cleavage pathway while *Pseudomonas*

acidovorans and *Pseudomonas testosterone* use the ortho and meta cleavage. (Nakazawa and Hayashi 1977, Eaton and Ribbons 1982, Ahuactzin-Perez *et al*; 2018 and Zhang *et al*; 2018).

In the case of anaerobic biodegradation of Phthalic acid, studies carried out through decarboxylation to benzoate. The key intermediate benzoate is then cleaved and biodegraded via the β -oxidation pathway to form H_2 , CO_2 , and CH_3COOH respectively. The formation of benzoate from Phthalic acid is the most critical step in the anaerobic biodegradation of PAEs (Liu and Chi 2003).

2.8. Enzymatic Biodegradation/Biotransformation of PAEs

Enzymatic treatment of organic contaminants has many advantages compared to conventional biological waste treatment methods. For example, enzymes are more resistant to toxic and shock loading effects, and there is no need for acclimation. In addition, enzymes could be applied to a wide range of organic compound concentrations, pH, temperature, and salinity. Bioremediation of polluted sites using enzymes may be very advantageous compared to classical methods in which degrading microorganisms are inoculated or stimulated in the polluted sites with the supplies of their nutrients since inoculation and control of microorganism to the environment is difficult and addition of nutrients to the polluted site may cause the increase of the chemical oxygen demand of the water environment (Zhang *et al*; 2018).

However, the application of enzymes to a polluted site, a process known as enzymatic bioremediation, will diminish those defects of the bioremediation by microorganisms. Enzymes could be isolated and applied to the polluted site and provide rapid remediation. Major enzymes that are involved in the metabolism of PAEs are phthalate oxygenase, phthalate dioxygenase, phthalate dehydrogenase, and phthalate decarboxylase (Nomura *et al*; 1992, Kurane *et al*; 1997). In addition, lipase and esterase enzymes from bacteria (Kurane *et al*; 1997) or animals were also used in the first step hydrolysis of PAEs that includes the formation of corresponding monoesters and alcohols. (Figure 4 & 5)

Kurane *et al*; (1997) conducted a study that involves enzyme induction from the *Nocardia erythropolis* and enzymatic hydrolysis of DEHP by commercial lipases from microbial sources. They reported the induction of phthalate ester-hydrolyzing enzymes from the *N. erythropolis* in the presence of 4000 mg/L DEHP. They also

suggested that phthalate esterase activity was observed in cells and cell-free extracts even microorganism was grown in medium containing olive oil as the sole source of carbon and energy. This finding suggested that phthalate esterase is a kind of lipase or esterase with broad substrate specificity. Removal ratio for DEHP by 1 mg/ml enzyme (30 °C and 18 h) microbial lipases from *Rhizopus delemar*, Steapsin (Pancreas), *Rhizopus arrhizus*, *Pseudomonas* and esterase from pig liver was found as 80.4, 24.8, 36.6, 19.1, 16.1% respectively.

Enzymatic degradation of DBP by lipase enzyme purified from *Candida cylindracea* was investigated in the study of Tanaka *et al*; 2006. In order to obtain model contaminated sediment, DBP was adsorbed on sea sand (1.5 µmol/0.5 g-seas and). After a 3-day incubation at pH 5, about 90% removal was obtained for DBP. The degradation of DBP was mainly due to the hydrolysis of ester bonds by the lipase enzyme. Fungal cutinase purified from *F. oxysporum* and commercial *Candida cylindracea* esterase enzymes were used for testing biodegradation of BBP. 500 mg/LBBP was mixed with enzyme solutions of cutinase and esterase and incubated 3 days in a shaking incubator (30 °C, 200 rpm). The cutinase enzyme (10 mg/L) degraded about 60 % of the BBP within 7.5 h. However, only 10% of the initial BBP was removed after 3 days incubation with esterase enzyme (Ejlertsson *et al*; 1996, Fang *et al*; 2010, 2017 and Kim *et al*; 2002).

In the study of Gu *et al*; (2005 and 1990), anaerobic degradation of primary sludge containing DEP, DBP, and DEHP as well as enzymatic degradation of these PAEs with commercial lipase was investigated. The sludge was pretreated at 70 °C and subsequent anaerobic biodegradation was applied at 37 °C. The pretreatment of sludge at high temperatures negatively influenced the biodegradability of PAEs. In addition, enzymatic treatment of the sludge with commercial lipase resulted in one to two orders of magnitude higher DEHP removal than under normal mesophilic conditions. The percent removal of DEHP (7 mg/L) after 100 h incubation at 28 °C with commercial lipase was about 85%.

Zeng *et al*; (2004) reported that hydrolysis of DEHP to PA by esterase from *Norcardia erythropolis*. The researchers suggested the initial degradation of DMP by esterase from *Bacillus* species. There are also some studies on PAE hydrolysis by mammalian enzymes, such as nonspecific lipase from rat pancreas (Chang *et al*; 2007), esterase from rat intestine and carboxylesterase from rat and human and salivary esterase from the human.

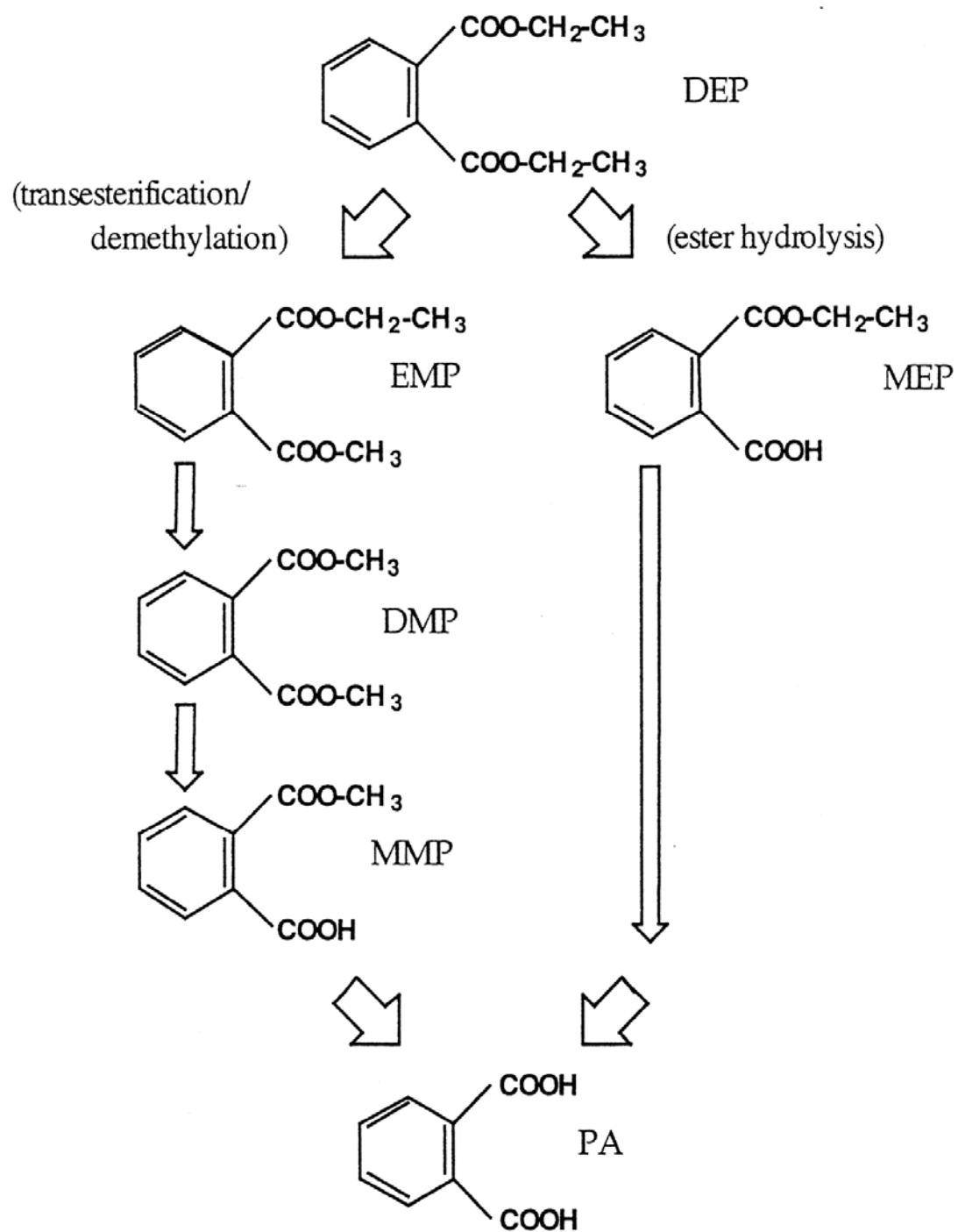
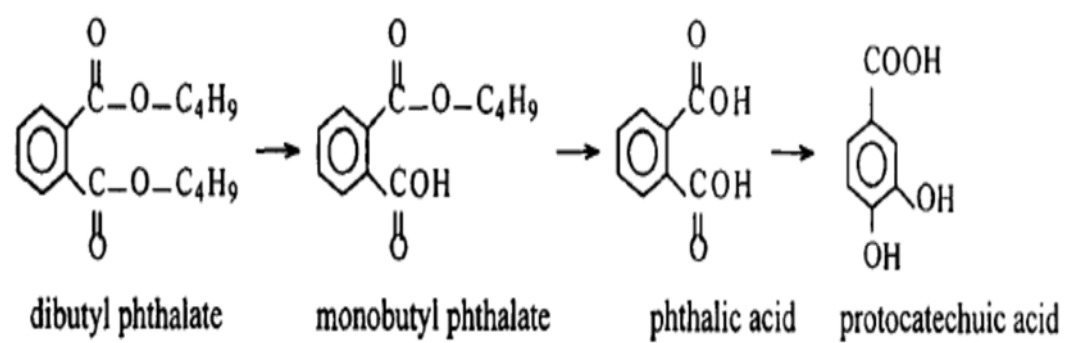


Figure 4: Biodegradation of DEP with formation of different intermediates



TCA cycle

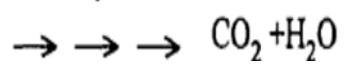


Figure 5: DBP degradation pathway into probable metabolites

Liang *et al*; (2008) reported that the primary degradation of PAEs consists of different types of pathways, including de-esterification or dealkylation, α -oxidation, and trans-esterification. De-esterification is the most common way of PAE degradation in which phthalate diesters are serially converted to phthalate monoester and phthalic acid. This degradation pathway is the same under both aerobic and anaerobic conditions (Shelton *et al*; 1984).

β -oxidation involves the biodegradation of phthalate diesters by removing one ethyl group at each time (Amir *et al*. 2005). After that, DEP is further converted to PA by two pathways, de-esterification and an alternative trans esterification pathway (demethylation) which is the replacement of the ethyl group of DEP with a methyl group.

Transesterification reaction normally requires a high temperature and pressure however in the presence of a biological catalyst it can proceed under ambient conditions. Demethylation reaction which is the replacement of each ethyl group with a methyl group followed by a transesterification reaction that results in cleavage of relatively strong C-C bond within the ethyl group is cleaved resulting in EMP and then DMP (Cartwright *et al*; 2000). Also studied hydrolysis of DEHP using commercial crude lipase from the porcine pancreas (Type II, 147 U). The researchers reported that incubation of 100 mg/L DEHP with 1470 U lipase solution at 37 °C for 24 h resulted in a 93% decrease in initial concentration. However, optimization of conditions for a better removal performance has not been studied for PPL. Enzymatic degradation studies on PAEs generally studied at high concentrations ranging from 5 to 2000 mg/L. (Figure 6)

Lipase and esterase enzymes were commonly used for the removal of PAEs since these enzymes are known to be effective on ester bonds. Degradation efficiency for PAEs ranged from 10 to 90% depending on the PAE concentration and the type of the enzyme used. Results of the studies indicate that the removal of PAEs by using enzymes is an effective way and there is no study on the application of thermophilic lipase enzyme for the degradation of PAEs.

2.9. Conclusion

PAEs are a group of refractory organic compounds and are one of the most frequently detected persistent organic pollutants in the environment. They are widely

used in the manufacture of a wide range of consumer able food packaging, plasticizers, some children's toys products, greenhouses, cosmetics, building materials, and some PVC and medical devices. PAEs can easily transfer into the environment from PVC products and other related by-products because PAEs are not strictly chemically attached to the polymeric matrix. Many researchers have reported that PAEs and their metabolites can impact reproduction, developmental and behavior of not only human beings but also on mammals, even at very minute concentrations. Hence, the EPA of many countries has categorized most PAEs as top priority pollutants. This review give a useful framework to identify future research objectives to achieve the mineralization and elimination of PAEs in the environment.

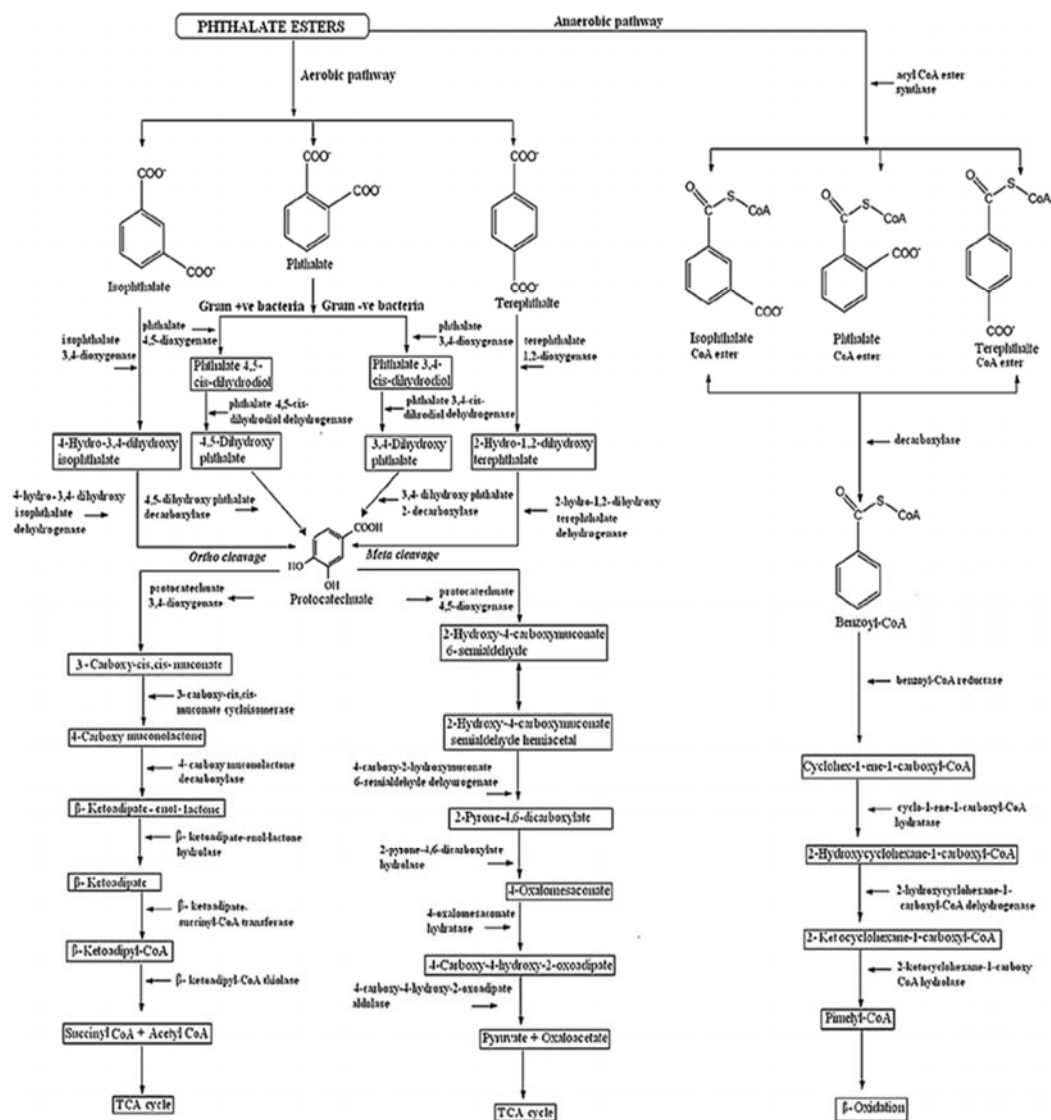


Figure 6: Bacterial metabolism of phthalate esters and linkages with metabolic pathways

3. MATERIALS AND METHODS

3.1. General

3.1.1. Location

The laboratory experiments were conducted at ICAR-Indian Agricultural Research Institute, New Delhi in mainly two divisions, Division of Microbiology and Division of Agricultural Chemicals. The location stands at 28.08°N and 77.12°E, the height above mean sea level being 229 m (or 750 ft).

The details of materials used and methodology followed during research work has been mentioned here under.

3.1.2. General procedure

In all the experimental studies, Borosil glassware was used. The glassware was kept for 24 h in chromic solution containing 60 g of potassium dichromate ($K_2Cr_2O_7$) and 60 ml of concentrated sulphuric acid (H_2SO_4) in one liter of water. Later glassware was cleaned by using the detergent followed by rinsing with distilled water and drying in hot air oven before use.

3.1.3. Sterilization

All the glassware was sterilized in a hot air oven at 160°C for three hours. All culture media, broth, and water blanks were sterilized in an autoclave at 15 lbs pressure and temperature 121°C for 20 minutes. Isolation, purification, inoculation and other microbiological work were carried out in laminar airflow chamber.

3.1.4. Chemicals and Reagents used

All the chemicals and reagents used were Analytical Reagent (AR) grade obtained from Hi Media Laboratories, Megazyme, Qualigens fine chemicals, E-Merck, BDH, Sigma, Fischer and Sisco Research Laboratories.

3.2. Microorganisms

3.2.1. Survey and soil sampling:

Phthalate esters affected soil samples were collected from three different sites at Centre for Cultivated Protection Technology (CPCT) IARI, New Delhi, India. A total 5 composite soil samples were collected at 0-15 cm depth using a soil auger. Each

composite sample was made from five sub samples which was collected along the zigzag paths (Zigzag sampling) to account for the randomness. The collected soil samples were properly labeled and stored in polythene bags and transported to the laboratory.

3.2.2. Soil physicochemical analysis

The soil physiochemical properties as described below were done as per the method described by Subbiah and Asija, (1956); Olsen (1952).

3.2.2.1. pH:

Soil samples were taken in conical flasks and shaken properly on gyratory shaker at 120 rpm for two hours. The pH of soil sample was determined in 1:2 (soil: water) suspension using combined electrode (glass and calomel electrodes) by digital pH meter.

3.2.2.2. Electrical Conductivity:

The electrical conductivity (EC) was determined in the supernatant liquid of the same extracts with the help of a conductivity bridge and expressed in dS m^{-1} at 25 °C.

3.2.2.3. Available N content:

Available Nitrogen content in soil was determined using Kjeltex Semi-Auto Nitrogen Analyzer by alkaline potassium permanganate method as proposed by Subbiah and Asija, (1956). The method has been widely adopted to get a reliable index of nitrogen availability in soil due to its rapidity and reproducibility.

Procedure: Five g soil sample was weighed and transferred in a distillation tube. The sample was moistened with 5 mL distilled water, washing down the soil adhering to the neck of tube. Twenty-five mL 0.32% KMnO_4 was added to it and the distillation tube was set to the instrument. In a 250 mL conical flask, 20 mL 2 % boric acid mixed indicator was taken and placed under the receiver tube. Tap water was run continuously in the condenser. Twenty-five ml 2.5% NaOH was sucked and added to the distillation tube. Then it was put on distillation for 9 min. During this process, the N released in the form of ammonia is trapped in the boric acid, which develops green colour. The flask containing the distillate was removed. The distillate was then titrated against 0.02N H_2SO_4 until pink colour developed.

3.2.2.4 Olsen phosphorus:

Available phosphorus content of soil was determined by Olsen's method (Olsen *et al*; 1954). Firstly, reagent A was prepared by using ammonium molybdate, antimony potassium tartrate, and H_2SO_4 . Then always reagent B was prepared fresh with the help of reagent A. Two-gram soil was taken in a 150 mL conical flask, a pinch of Darco G-60 phosphorus free carbon powder and 40 mL Olsen's reagent (0.5 M NaHCO_3) was added to it. It was then shaken for 30 minutes on mechanical shaker and the suspension was filtered through Whatman no. 1 filter paper. Five ml filtrate was transferred in a 25 mL volumetric flask and was acidified with 2.5 M H_2SO_4 to pH 5.0 and 20 mL distilled water was added followed by 4 mL reagent B. After waiting for 10 min the intensity of blue colour was measured on spectrophotometer at 882 nm. Simultaneously a blank was also run. First standard reading was taken followed by sample reading.

3.2.2.5. Available potassium (Black *et al*; 1965)

Potassium in soil exists as water soluble, exchangeable, non-exchangeable and lattice form. Ammonium acetate method was followed for determination of available potassium. Five gram soil sample was weighed in 100 mL conical flask. 25 mL neutral 1N ammonium acetate solution was added to it and shaken for 5 min on the mechanical shaker. It was filtrated through Whatman no. 1 filter paper. Potassium concentration was measured using flame photometer. Standard solutions of 10, 20, 30, 40 and 50 ppm were prepared by using KCl solution.

3.2.2.6. Available sulphur

Available sulphur content in soil was determined by turbidimetric method using 0.15% CaCl_2 -extractant (Black *et al*; 1965). Five gram soil was taken in a 100 mL conical flask and 25 mL 0.15% CaCl_2 solution was added into it. The content was shaken for 30 minutes on a shaker and the suspension was filtered through Whatman no. 42 filter paper. Then 10 mL aliquot was transferred to a 25 mL volumetric flask and 1 gm sieved BaCl_2 crystals were added and it was shaken for 1 minute. One ml 0.25% gum acacia solution was added and the volume was made to the mark. It was shaken for one minute and the turbidity was measured after 25 to 30 minute on spectrophotometer at 420 nm wavelength. Simultaneously a blank was also carried following same procedure. First standard reading was taken followed by sample reading.

3.2.2.7. Available boron

Available B content of soil was determined by Hot CaCl_2 - extractable method and boron in the extracts was determined by Azomethine-H method (Wolf, 1971). Five mL sample aliquot, 2 mL ammonium acetate buffer (pH 5.5) and 2 mL 0.02 M EDTA were added to a 20 mL B free test tube and vortexed. After adding 1 mL azomethine-H reagent (0.9% azomethine H + 2% ascorbic acid solution) the tube was again vortexed, allowed to stand for 1 h at $\sim 25^\circ\text{C}$ and vortexed again, and the readings were taken at 420 nm using the spectrophotometer.

3.2.2.7. Available zinc, copper, iron, and manganese

Available zinc (Zn), copper (Cu), iron (Fe) and manganese (Mn) were determined by extracting soil sample with DTPA-extractant as outlined by Lindsay and Norvel, (1978) Ten gram air-dried soil + 20 mL extracting solution were shaken during 2 h. Soil extracts (2:1 soil solution ratio) were obtained with DTPA-TEA (0.005M diethylenetriaminepentaacetic acid + 0.1M triethanolamine + 0.01 M CaCl_2) solution at pH 7.3 as described by Lindsay and Norvell, (1978) and in the content of these metals in the extracts were estimated by an atomic absorption spectrophotometer (Agilent FS-240).

3.2.2.9. Organic carbon:

Organic carbon content in soil was determined by wet oxidation method as outlined by Walkley and Black (1934). Soil organic matter is the seat of nitrogen in soil and its determination is often carried out as an index of nitrogen availability. One gm (0.2 mm) soil samples added into 500 mL dry conical flask of borosilicate glass. 10 mL 1N $\text{K}_2\text{Cr}_2\text{O}_7$ and 20 mL concentrated sulfuric acid was added in the soil sample. Swirled the flask a little and kept on an asbestos sheet for 30 minutes. Slowly added 200 mL distilled water followed by 10 mL orthophosphoric acid and finally 1 mL diphenylamine indicator. 0.5N ferrous ammonium sulfate solution was taken in 50 mL burette. Titrated the contents until green colour started appearing. One mL 0.2 N ferrous ammonium sulfate is equivalent to the 0.009807 gm $\text{K}_2\text{Cr}_2\text{O}_7$ or 0.0006 gm carbon. Organic carbon content in the sample was calculated as:

$$\text{Organic carbon (\%)} = (B - S) \times 0.0006 / m \times 100$$

Where B is the volume of ferrous solution used in the blank titration, S is the volume of ferrous solution used in the sample titration, m is the mass of the sample in gm used in the analysis. No correction factor was applied to the OC content calculation.

3.3. Residual phthalates esters in soil using HPLC

Hundred-gram soil was taken in 250 mL conical flask. Added 10 mL water followed by 50 mL acetone. Kept the flasks on shaker for half an hour and then filled it. Again, added 10 mL acetone in the flask and filtered once again. Evaporated acetone using rotary evaporimeter after complete extraction. Added 50 mL aqueous NaCl (20%) to the residue and using separating funnel partitioned the contents with 50 mL di chloro methane (DCM). The lower layer was collected and again partitioned with 30 mL DCM and same procedure was repeated with 10 mL DCM also and further DCM was evaporated using rotary evaporimeter.

Finally, 2 mL methanol was added in the remaining residue in rotary evaporimeter and all the contents were filtered using a 0.22 micron filter. The sample was estimated using HPLC.

3.3.1. HPLC conditions:

HPLC analysis was carried out on a Hewlett Packard instrument (series 1100) equipped with degasser, quaternary pump, diode array detector (DAD) connected with Rheodyne injection system (20 μ L loop) and a computer (model Vectra). The stationary phase consisted of Lichrospher on C – 18 packed stainless steel column.

1. Column - Lichrospher RP – 18 (250 mm x 4 mm id)
2. Mobile phase - Methanol – Water (80:20) (Isocratic)
3. Flow rate – 1mL/min
4. Injection volume - 20 μ L
5. Wavelength - 246 nm
6. Retention time (RT) - 2.45 – 5.83 min for PAEs

Residual PAEs

$$= \frac{\text{Peak area of extracted sample} \times \text{concentration of stand.} \times \text{vol. of extract}}{\text{Peak area of standard} \times \text{volume of sample}}$$

3.4. Enrichment of soil samples

Enrichment of soil samples was done with DEP and DEHP at appropriate concentrations for 6 weeks using minimal salt (MS) medium having both PAEs ($50 \mu\text{g mL}^{-1}$).

Hundred grams soil from each sample was transferred aseptically to 250 mL conical flask containing 90 mL MS medium having PAEs @ $50 \mu\text{g mL}^{-1}$ and incubated on rotary shaker at 30°C for 7 days at 200 rpm. After a week's incubation, 10 mL soilsuspension from flask was transferred aseptically to another 250 flask having fresh sterile 90 ml MS medium with PAEs @ $50 \mu\text{g mL}^{-1}$. Flask was again incubated for 7 days and same step was repeated 4 more times.

The composition of MS medium (Pfennig and Lippert, 1966) is as follow

Composition of MS Medium

Ingredients	Quantity (g L^{-1})
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.02
KH_2PO_4	0.68
K_2HPO_4	1.73
$(\text{NH}_4)_2\text{SO}_4$	0.017
Trace element solution	1 mL
Distilled water	1000 mL

Trace element solution composition:

Ingredients	Quantity (ppm)
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	200
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	10
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	3
H_3BO_3	30
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	1
$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	2
Na_2MoO_4	1

PAEs stock solutions were prepared by dissolving 300 μ L PAEs in 100 mL ethanol and filter sterilized using 0.45 μ m filter. PAEs were added as and when required in sterilized MS medium as sole C source at desired concentration.

3.4.1. Isolation of bacteria from enriched sample:

After 6 weeks of enrichment, bacterial strains from enriched soil samples were isolated by serial dilution technique and purified by repeated streaking on MS medium with PAEs @ 50 μ g mL⁻¹ agar plates. All the purified isolates were subjected to morphological characterization and observations were recorded for viz. colony color, size, pigmentation, margin and elevation after 48 h of incubation. Representative morphotype was purified, sub-cultured and maintained on MS medium agar slants having the same PAEs @ 50 μ g mL⁻¹.

3.5. Microorganisms

In the present investigation all microorganisms mainly bacteria were isolated from phthalate contaminated soil and eighteen soil bacterial isolates (Table 3.1) were selected for further studies.

3.6. Total soluble protein estimation (Lowry *et al.*, 1951)

Reagents:

3.6.1. Alkaline copper reagent (ACR):

Hundred mL NaOH (0.1N) was prepared and 2.0 g Na₂CO₃ was added in NaOH solution. Two mL solution discarded from the above solution. Then 1.0 mL Na-K-tartrate solution (1.0% w/v) was added in 98 mL Na₂CO₃ containing NaOH solution, followed by 1.0 mL freshly prepared CuSO₄ solution (1% w/v). ACR solution was prepared fresh every time.

3.6.2. Folin Ciocalteu's reagent: Commercially available reagent (2N) was diluted to 1N with addition of equal volume of distilled water.

3.6.3. Estimation method:

Cell suspension (0.5 mL) was added to test tube containing 5 mL alkaline copper reagent and incubated for 10 min at room temperature. After 10 min, 0.5 mL, 1N Folin Ciocalteu's reagent was added to the tubes and these were again incubated

for 30 min at room temperature. A blank was also prepared with 0.5 mL distilled water instead of sample. Absorbance was taken at 750 nm. The protein concentration was estimated by referring to standard prepared with Bovine serum albumin (10-100 $\mu\text{g/mL}$).

3.7. Cell pellet protein estimation (Lowry *et al.*, 1951)

3.7.1. Reagents:

1. Alkaline copper reagent (ACR):

For preparation of ACR same protocol was followed as for the total protein estimation.

2. Folin Ciocalteu's reagent:

As per the protocol is given in the total protein estimation the Folin's reagent was prepared.

3. 2N NaOH:

8.0 g NaOH was dissolved in 100ml sterilized distilled water.

4. 2N HCl:

6.2 mL HCl was dissolved in 93.8 ml sterilized distilled water.

Estimation method:

One mL cell suspension was centrifuged at 10000 rpm for 5 min at normal room temperature. The obtained cell pellet was dissolved in 0.5 mL 2N NaOH. The obtained suspension was boiled at 100°C for 30 min. After that, the tubes were cooled and 0.5 mL 2N HCl was added. 0.5 mL aliquot was taken and added 0.5 mL sterilized distilled water. 5 mL alkaline copper reagent was added to the pellet suspension and incubated for 10 min at room temperature. After 10 min, 0.5 mL, 1N Folin Ciocalteu's reagent was added to the tubes and these were again incubated for 30 min at room temperature. A blank was also prepared with 0.5 mL distilled water instead of sample. Absorbance was taken at 750 nm. The protein concentration was estimated by referring to standard prepared with Bovine serum albumin (10-100 $\mu\text{g/mL}$).

Table 3.1: List of selected PAEs degrading bacterial isolates

S.No.	Name of culture	Place of origin
1	DEP A1	CPCT, IARI
2	DEP A2	CPCT, IARI
3	DEP A3	CPCT, IARI
4	DEP B1	CPCT, IARI
5	DEP B2	CPCT, IARI
6	DEP B3	CPCT, IARI
7	DEP C1	CPCT, IARI
8	DEP C2	CPCT, IARI
9	DEP C3	CPCT, IARI
10	DEHP A1	CPCT, IARI
11	DEHP A2	CPCT, IARI
12	DEHP A3	CPCT, IARI
13	DEHP B1	CPCT, IARI
14	DEHP B2	CPCT, IARI
15	DEHP B3	CPCT, IARI
16	DEHP C1	CPCT, IARI
17	DEHP C2	CPCT, IARI
18	DEHP C3	CPCT, IARI

3.8. Preparation of standard curve for protein using BSA

From 1 mg/mL stock solution of BSA, different volumes 50,100,150, 200, 250, 300, 350, 400 and 450 μ L were taken in a test tube and final volume was made up to 1 mL using distilled water. Five ml alkaline Copper Reagent was (prepared by mixing 50 mL 2% (w/v) Na_2CO_3 solution in 0.1N NaOH and 1 mL 0.5% (w/v) copper sulphate solution in 1 % (w/v) sodium potassium tartarate solution) added in all the tubes and the tubes were incubated at room temperature for 10 minutes. 0.5 mL 1N Folin-Ciocalteu's Reagent (prepared by dilution of the commercially available Folin-Ciocalteu's reagent (2N) with equal volume of distilled water) was added followed by incubation at room temperature under dark condition for 30min. The absorbance was read at 660 nm. Three replicates of each dilution were taken. This absorbance along with concentration was plotted to get the standard curve. Concentration of unknown sample was determined by referring to the standard curve.

3.9. Microbial growth in terms of absorbance at 600 nm

Estimation method:

One mL cell suspension in the glass cuvette was taken for measuring absorbance at 600 nm and MSM medium was kept as blank.

3.10. Hydrolytic enzyme production profiling of bacterial isolate using API-ZYM kit

The API-ZYM kit (Bio Merieux, France) specially designed for the study of enzymatic reactions was used which has the strip with base already containing synthetic substrates made of non-woven fibers. This base allows enzymatic reactions to take place, even if the substrates are insoluble. The bacterial cultures raised in TY broth were inoculated in the cupules in the base strips of API-ZYM kit and incubated at 37° C for 4 hours. After incubation ZYM-A and ZYM-B reagents were added (provided with the kit) to the cupules. The strips were put under a powerful light source (1000 W bulb) for 10 sec and were read for colour reactions according to Table

3.10.1. Functional Characteristic as tested by API-ZYM strip

No.	Enzyme assayed for	Substrate	pH	Results	
				Positive	Negative
1	Control			Colourless or color of the sample if it has an intense coloration	
2	Alkaline phosphatase	naphthyl phosphate	8.5	Violet	Colourless or very pale yellow
3	Esterase (C 4)	2-naphthyl butyrate	6.5	Violet	
4	Esterase Lipase (C 8)	2-naphthyl caprylate	7.5	Violet	
5	Lipase (C 14)	2-naphthyl myristate	7.5	Violet	
6	Leucinearylamidase	L-leucyl-2-naphthylamide	"	Orange	
7	Valinearylamidase	L-valyl-2-naphthylamide	"	Orange	
8	Cystinearylamidase	L-cystyl-2-naphthylamide	"	Orange	
9	Trypsin	N-benzoyl-DL-arginine-2-Naphthylamide	8.5	Orange	
10	α -chymotrypsin	N-glutaryl-phenylalanine-2-Naphthylamide	7.5	Orange	
11	Acid phosphatase	2-naphthyl phosphate	5.4	Violet	
12	Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	"	Blue	
13	α -galactosidase	6-Br-2-naphthyl-aD-Galactopyranoside	"	Violet	
14	β -galactosidase	2-naphthyl-IID-Galactopyranoside	"	Violet	
15	β -glucuronidase	Naphthol-AS-BI-IID-Glucuronide	"	Blue	
16	α -glucosidase	2-naphthyl-aD-Glucopyranoside	"	Violet	
17	β -glucosidase	6-Br-2-naphthyl-BD-glucopyranoside	"	Violet	
18	N-acetyl-B-glucosaminidase	1-naphthyl-N-acetyl-BD-glucosaminide	"	Brown	
19	α -mannosidase	6-Br-2-naphthyl-CLD-mannopyranoside	"	Violet	
20	α -fucosidase	2-naphthyl-CLI-fucopyranoside	"	Violet	

*Colorless or color of the control if the strip has been exposed to an intense light source after addition of the reagents: if the strip has not been exposed to intense light, a very pale yellow color is obtained.

3.11. Biochemical characterization of the isolates

Based on morphological characterization, selected bacterial isolates were tested for their positive or negative response to different biochemical tests on the basis of which they were compared to different representative genera in Bergey's manual of determinative bacteriology. The various biochemical tests performed and the method to detect these biochemical parameters are given as under: (Cappuccino *et al*;1992)

3.11.1. Gram Staining:

Bacterial smear was prepared from actively growing cells on a glass slide and fixed by heat. Flooded Crystal violet for 10 sec and washed with tap water to remove excess crystal violet. Gram iodine was flooded for 10 sec and again washed briefly in tap water. Further, it was decolorized with ethyl alcohol until the moving dye front has passed the lower edge of the section and washed immediately in tap water. Safranin was used to counter stain for 15 sec and washed with water to remove the excessive stain. The slides were visualized under microscope at different magnifications after drying.

3.11.2. Catalase test:

A loopful of culture from freshly grown slant of tryptone-yeast extract medium was taken out and placed on the slide. A drop of H₂O₂ (3%) was added to growth. Production of bubbles indicated presence of catalase. The medium used was Tryptone Yeast Extract Broth and its composition is as follows:

Tryptone Yeast Extract Medium (TY) (Beringer, 1974)

Ingredients	Quantity(gm L ⁻¹)
Bacto-tryptone	5.0
Yeast Extract	3.0
CaCl ₂	0.66
pH	7.0

3.11.3. Cytochrome oxidase:

Oxidase reagent (Kovac's) was added to colonies growing on solid TY medium. Development of purple colour indicated the presence of oxidase.

Kovac's reagent for oxidase test (Bailey and Scott, 1960)

Ingredients	Quantity(gm L ⁻¹)
p-dimethyl amino-benzaldehyde	10.0
Amyl alcohol	150.0
HCl (conc.)	25.0
pH	7.0

The p-dimethyl aminobenzaldehyde was dissolved in the amyl alcohol, HCl was then added and kept in a glass stopper bottle and was stored in refrigerator.

3.11.4. Nitrate reduction:

Nitrate broth was inoculated with test cultures and incubated at 35°C for 48 h. To test for the presence of nitrite, Griess Ilosvay's reagent was added to the tubes and development of pink colour indicated nitrate reduction. To test complete denitrification a pinch of zinc dust was added to the tubes, reappearance of pink colour indicated complete denitrification.

Nitrate-Broth (Bailey and Scott, 1966)

Ingredients	Quantity (gm L ⁻¹)
Peptone	5.0
Beef Extract	3.0
Potassium Nitrate	5.0
pH	7.2

3.11.5. Urease test:

Urea broth containing phenol red indicator was inoculated with test cultures and incubated for 5 - 6 days at 37°C. Presence of yellow colour indicated the presence of urease.

Urea Agar (Christensen, 1946)

Ingredients	Quantity(gm L⁻¹)
Peptone	1.0
Dextrose	1.0
NaCl	5.0
Na ₂ PO ₄	1.2
KH ₂ PO ₄	0.8
Phenol red	0.012
Urea*	20.0
Agar	16.0

*The medium was autoclaved and cooled to 55 °C and 50 mL of filter-sterilized urea solution (40%) was added. The pH of the medium was adjusted to 6.8-7.0.

3.11.6. Starch hydrolysis:

Starch plates were prepared and streaked with test cultures. Plates were incubated at 30°C for 24 h. Following incubation, plates were flooded with Gram's iodine. Zone of clearing around growth showed starch hydrolysis.

Starch Agar (Cappuccino and Sherman, 1992)

Ingredients	Quantity(gm L⁻¹)
Peptone	5.0
Beef Extract	3.0
Starch	2.0
pH	7.0

3.11.7. Lipase Activity:

Agar medium supplemented with 0.01% CaCl₂.H₂O is used for this test. Tween 80 sterilized by autoclaving at 121° C for 20 minutes. The sterilized Tween 80 was added to the molten agar medium at 45 to 50° C to give a final concentration of

1% (v/v). The medium was stirred. After the solidification, the plate was streaked and checked for an opaque halo around the colonies. Microscopically, the halo consists of crystals of calcium soaps.

Modified medium for lipolytic activity (Sierra, 1957)

Beef extract	3.0
Peptone	5.0
NaCl	5.0
Tween- 80	1% vol/vol
CaCl ₂ .H ₂ O	0.1
Agar	18.0
pH	7.0-7.2

3.12. Screening of bacteria for maximum PAEs tolerance

All the selected morphotypes were screened for their ability to grow at 100, 300, 500 and 700 ppm PAEs in MS broth. Desired concentration of filter-sterilized PAEs from its stock solution was added to sterile MS broth in order to get final concentration 100, 300, 500 and 700 ppm. The flasks were incubated at 30°C at 200 rpm for 48 h. After 48 h of incubation pH of broth was measured using pH meter and total protein was estimated.

3.13. 16S rRNA based Molecular Characterization of bacterial isolates

3.13.1. Isolation of total cell DNA from bacterial isolate (Modified method of Carles and Nester, 1993)

Reagents:

Tryptone Yeast Extract Broth Medium

Tryptone	1.0 g
Yeast Extract	5.0 g
NaCl	5.0 g
Glucose	1.0 g

Tris-EDTA buffer (T₁₀E₂₅)

For preparing 100 mL T₁₀E₂₅ buffer, added 2 mL Tris-HCl stock and 5 mL 0.5 mM EDTA stock were and mixed the volume up to 100 mL was prepared.

Tris-HCl Stock Solution (pH 7.5)

Dissolved 12.1 g Tris (Hydroxy methyl amino methane)-HCl in 75 mL sterile double distilled water. Added concentrated HCl drop wise till pH 7.5 was attained. Final volume to 100 mL with double distilled water was prepared.

EDTA Stock solution (0.5M)

18.612 gm EDTA disodium salt was added in 40 mL of sterile double distilled water in a pre-sterilized reagent bottle. Placed the bottle on a magnetic stirrer for uniform shaking and slowly added 40% NaOH solution through the wall of reagent bottle till pH 8.0 was attained. 100 mL was the final volume attained with sterile double distilled water.

Sodium Dodecyl Sulphate solution (25%)

Dissolved 25 g Sodium dodecyl sulphate (Sodium lauryl sulphate) in 75 mL sterile double distilled water in a clean, pre-sterilized reagent bottle by slowly stirring and avoiding formation of broth and 100 mL was prepared as the final volume.

Lysozyme stock solution (20 mg/mL)

Dissolved 0.2 g Lysozyme powder in 10 mL sterile milliQ water and stored at -20⁰ C for future use.

Ammonium acetate solution (7.5 M)

57.81 g ammonium acetate was added in 50 mL sterile double distilled water in a pre - sterilized reagent bottle, stirred till homogeneous, transparent solution was obtained and 100 mL was prepared as the final volume.

Method

Total cell DNA of bacteria was isolated by the method of Charles and Nester, 1993 with slight modifications. Pure culture of bacteria was raised in 5 mL TY medium broth for 18 – 24 hrs to obtain cell O.D. 0.6 at 600 nm. The culture broth (1.5 mL) was pelleted in a microfuge tube for 2 min. at 12000 rpm. The bacterial pellet

was washed in 1.5 mL 0.85% NaCl, centrifuged for 2 min. at 12000 rpm and was resuspended in 0.4 mL Tris-EDTA buffer($T_{10}E_{25}$).

Cell lysis was done by adding 20 μ L 25% SDS, 50 μ L 1% lysozyme and 50 μ L 5M NaCl followed by incubation at 68⁰ C for 30 min. in a circulatory water bath. For protein precipitation, 260 μ L 7.5 M Ammonium acetate solution was added to the microfuge tubes and the tubes were kept in ice for 20 min. followed by centrifugation at 13000 rpm for 15 min. at 20⁰ C. Supernatant was carefully pipetted out in another fresh, sterile microfuge tube in which 1 μ L RNase (4 mg/mL) was added followed by incubation at 37⁰ C for 20 min. Equal vol. of chloroform was added in the tubes and proper mixing was done by inverting the tube up and down several times. RNA was precipitated by centrifuging for 1 min. at 12000 rpm. The top layer containing total cell DNA was pipetted out in fresh microfuge tube and used for next step. DNA was precipitated by adding 0.8 ml isopropanol followed by incubation on ice for 30 min. and pelleted by centrifuging at 10000 rpm for 15 min. DNA was further washed with 0.5 mL 70% Ethanol and spun down at 10000 rpm for 1 min. Traces of Ethanol were removed by air drying the tubes in inverted position. Pure DNA sample was then suspended in 20 mL Tris-EDTA buffer ($T_{10}E_1$) and stored at 4⁰ C for further use.

3.13.2. Agarose Gel Electrophoresis of Genomic DNA samples

Materials

Tris Borate EDTA (TBE) Buffer (5X Stock)

Dissolved 54 gm Tris-HCl, 27.5 gm Boric acid and 20 mL 0.5 M EDTA (pH 8.0) in 500 mL sterile MilliQ water and the final volume upto one-liter sterile MilliQ water was prepared.

TBE Buffer (0.5X working solution)

Mixed 100 mL 5X stock of TBE buffer in 500 mL sterile MilliQ water and the final volume upto one Liter with sterile MilliQ water was prepared.

Agarose (0.8%)

Dissolved 0.8 g agarose in 100 mL 0.5X TBE buffer in a 250 mL sterile Erlenmeyer flask, to homogeneity by melting in a microwave oven. When the

temperature of the solution was about 50-55⁰C added 3 mL ethidium bromide (1 µg/mL) and poured into the gel casting tray with combs in place. The agarose was allowed to polymerize for about an hour.

3.13.3. Quantification of genomic DNA

3.13. 3.1. Quantification through Spectrophotometric method

The genomic DNA was diluted 100 times and quantified by measuring the absorbance at 260 nm. The amount of DNA was estimated using the relationship that O.D. 1.0 corresponds to 50 µg/mL. The purity of DNA was assessed by measuring the A₂₆₀/A₂₈₀ ratio.

3.13.3.2. Quantification through Agarose Gel Electrophoresis

The genomic DNA sample of bacteria was quantified through agarose gel electrophoresis by analyzing their migration on 0.8% agarose gels prepared in 0.5 M Tris-borate-EDTA (TBE) buffer and run in an Electrophoresis tank filled with TBE buffer of the same conc. The genomic DNA was diluted with Tris-EDTA (T₁₀E₁) buffer so as to achieve a concentration 50 ng in 10 µL to be used as a template DNA in PCR amplification reaction.

Solutions and buffer for polymerase chain reaction (PCR)

All the chemicals were obtained either from Fermentas, U.K. or Bangalore Genei Pvt. Ltd., Bangalore, India.

- (a) Polymerase reaction buffer (10X): The polymerase reaction buffer commercially provided contains 100 mM Tris. HCl (pH 9.0); 500 mM KCl, 15 mM MgCl₂ and 0.1% gelatin.
- (b) Taq DNA polymerase: Taq DNA polymerase was 5U µL⁻¹ in a storage buffer consisting of 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20 (v/v), 0.5% Igepal and 50% Glycerol (v/v).
- (c) dNTP's mix: A stock of dNTP mix (dATP, dCTP, dGTP and dTTP) has 2.5 mM concentration of each dNTP.
- (d) Primers: The forward (PA) and reverse (PH) primers were custom synthesized from Sigma, USA. The sequence of the oligonucleotide primers used for amplification of 16S rDNA genes are:

PA: 5' CACGGATCCAGAGTTTGTATYMTGGCTCAG 3'

PH: 5' GTGCTGCAGGGTTACCTTGTTACGACT 3'

The stock solution (100 ng/mL) of the primers was prepared by reconstituting lyophilized primers in sterilized milli Q water and stored at -20°C.

3.13.4. Amplification of 16 S rDNA gene from the bacterial isolates

Method:

Amplification of 16S rDNA was carried out by polymerase chain reaction using a thermal cycler (M.J. Research PTC-100). The PCRs were carried out with 50-90 ng pure genomic DNA. The amplification reactions were performed in 100 µL mixture containing 0.6 U Taq DNA Polymerase (Genei from 5U/µL), 2.5 µL 10X Taq Polymerase buffer, 0.4 mL Dntp mix and 0.3 µL each of the two primers described above.

The Following programme was used for the amplification of 16 S rDNA.

Step 1: 94 °C for 5 minutes

Step 2: 94 °C for 30 seconds

Step 3: 65 °C for 30 seconds

Step 4: 72 °C for 1.30 minutes, Repeated step 2 to 4, 30 times (30 cycles)

Step 5: 72 °C for 10 minutes

Step 6: 4 °C forever.

For every PCR reaction, a negative control (no template DNA) and a positive control (template DNA giving amplified product) were invariably maintained. The amplified product was run on a 1.2 % agarose gel along with 1 kb MW marker, at a constant voltage, and visualized under UV light.

3.13.4.1. Purification of amplified PCR product

In order to remove traces of reagents used during amplification reaction the amplified 16S rDNA obtained after PCR amplification were purified and concentrated using Qiaquick PCR purification kit, obtained from Qiagen (Germany). The kit included Qiaquick spin columns, buffers PB and PE, and elution buffer. The protocol designed for purification renders DNA fragments ranging from 100 bp to 10 kb purified from primers, nucleotides, polymerases, and salts. 100µLPCR reaction was

mixed with 500 μ L buffer PB. Qiaquick spin column were placed in a provided 2 mL collection tube. The total sample (600 μ L) was applied to the column and centrifuged at 13,000 rpm for 30-60 seconds. The flow-through was discarded. The column was placed back on 2 mL collection tube and washed with 750 μ L buffer PE and again centrifuged for 30-60 seconds. The flow-through was discarded and the column was centrifuged for an additional one minute at 13,000 rpm. The Qiaquick column was placed on a clean 1.5 mL microfuge tube. To elute DNA, 50 μ L of milli Q water was applied at the centre of the Qiaquick membrane and the column was centrifuged for 1 minute. To get rid of nonspecific amplification of other undesired genes in some of the PCR products, the amplified product was purified through Gel elution kit procured from Genei. The eluate (approximately 48 μ L) was stored at 4°C. Double pass sequencing of the amplified PCR product of 16S rRNA gene was done through ABI-PRISM sequencer using Big Dye (Chromas Inc.).

3.13.5. Sequence analysis of the amplified PCR product of the selected isolates

The partial sequences of 16S rRNA gene of the bacterial isolate obtained after sequencing was analyzed using NCBI BLAST tool for identification of isolate. The query sequences (partial 16S rRNA gene sequence of bacterial isolates) were locally aligned against non-redundant (nr) nucleotide sequence database of NCBI using 'blastn' algorithm program for phylogenetic tree preparation (based on UPGMA algorithm) and comparison of the gene sequence of isolates with those of other phylogenetically close microbial relatives.

3.14. Recovery of phthalate esters from soil:

Ten gram soil sample was taken in 250 mL conical flask. The soil sample was fortified with PAEs @ 1 ppm and added 1 mL water. The extraction was initiated with acetone and 30 mL acetone was added to the sample, mixed thoroughly by shaking for half an hour on mechanical shaker. After the settling down of the soil, the supernatant was decanted on filter paper and collected in the conical flask. Again, the residue was treated with 15 mL acetone with 15 minutes shaking followed by decantation and filtration. Same procedure was followed using 5 mL acetone with manual shaking of 5 minutes. The collected supernatant was treated with 15% aq. NaCl and poured in a separating funnel, followed by addition of 50 mL DCM. The separating funnel was used for partition and collected the lower layer for further use.

Same procedure was repeated using 30 mL followed by 10 mL DCM and lower layer was collected in each steps. The whole collected sample was taken to rotary evaporimeter where DCM was evaporated and the residue was further treated with 2 mL methanol and filtered through 0.22 micron filter. The final titrate was estimated using HPLC.

Residual PAEs

$$= \frac{\text{Peak area of extracted sample} \times \text{concentration of stand.} \times \text{vol. of extract}}{\text{Peak area of standard} \times \text{volume of sample}}$$

3.15. HPLC analyses for DEP degradation by selected isolates in MSM broth

3.15.1. Preparation of mother culture:

The selected isolates were inoculated in 50 mL MS broth containing 100 ppm DEP and kept an incubator shaker at $30.0 \pm 2^\circ\text{C}$ for 48 hrs. The bacterial count was enumerated using serial dilution technique for each isolate.

3.15.2. Preparation of DEP stock solution:

Three grams DEP was dissolved in 100 MS broth for preparing 30000 ppm stock solution (3g/100 mL). Transferred 1 mL DEP from the stock solution in 100 mL MS broth in 250 ml conical flask. Each flask was inoculated with 5 ml bacterial isolates and incubated at 30°C in incubator shaker at 200 rpm. The samples were collected at 10, 20 and 30 days interval and analyzed further. The pH of medium was maintained at 7.0 ± 0.2 and the bacterial count was $6.0 \times 10^5 \text{ mL}^{-1}$ - $2.87 \times 10^6 \text{ cfu/mL}$. Degradation study was carried out for each isolate singly as well as in combination with each other, in other words, consortium study was also carried out.

3.15.3. Estimation of Residual DEP:

All the samples collected at 10, 20 and 30 days of incubation were analyzed for residual DEP. Each flask containing 100 mL MS broth and 5 mL bacterial culture after the desired incubation was taken for further analysis. Added 50 mL 15 % aqueous NaCl followed by 50 mL DCM. The contents were poured in separating funnel and after thorough mixing, lower layer was collected after filtration through sodium sulfate so as excess of water is adsorbed. Same procedure was repeated using 30 mL and 10 mL DCM in separating funnel. DCM from the filtrate was evaporated using rotary evaporimeter. Finally, the dried DEP sample was dissolved in 2.0mL methanol (HPLC

grade) and filtered through cellulose nitrate membrane (0.45 μ). Ten μ L sample was analyzed with Waters 2487 HPLC equipped with a UV-VIS (Waters-2487) detector set at wavelength of 254 nm using reverse phase C-18 column (size 250 x 4.6 mm) with particle size 5mm to carry out separation. The isocratic mobile phase was methanol: water (80:20 v/v) and flow rate were 1mL min⁻¹. The DEP concentration was calculated on the basis of peak area measurements by comparison with an external standard of known concentration of DEP (300 ppm) prepared in methanol. Finally, residual DEP was calculated similarly like residual PAEs and the degradation was calculated as:

$$\% \text{ DEP Degradation} = \frac{A_0 - A_n}{A_0} \times 100$$

where, A_n = Amount recovered (μ g mL⁻¹ or μ g g⁻¹) on n days; A_0 = Amount recovered (μ g mL⁻¹ or μ g g⁻¹) on 0 day; n= Time interval after fortification (i.e. 10, 20, 30.....days

3.16. Degradation of DEP in microcosm

Soil with no history of DEP accumulation was collected from IARI farm. The collected soil was sandy loam in texture total organic carbon, 58.06 kg ha⁻¹ available N, 10.38 kg ha⁻¹ available P and 105.25 kg ha⁻¹ exchangeable K. The soil was passed through 0.2 mm sieve. The sterile soil was fortified with analytical grade DEP (SIGMA-ALDRICH Inc) under aseptic conditions to obtain final concentration of 300 ppm (300 mg Kg⁻¹) in each pot.

Each pot was filled with 100 gm fortified soil at 30 mg DEP/ 100 gm soil. Sterile water was added in each pot for moistening the soil. The mother culture was prepared in 50 mL MS broth containing 100 ppm DEP of each isolate. 5 mL bacterial culture was transferred singly as well as in combination in each pot and incubated for 10, 20 and 30 days interval.

Following treatments were taken with three replications for each:

T1: Culture 1 + soil with 300 ppm of DEP

T2: Culture 2 + soil with 300 ppm of DEP

T3: Culture 3 + soil with 300 ppm of DEP

T4: Culture 1 +2 + 3 + soil with 300 ppm of DEP

T5: Un-inoculated control.

Where, Culture 1: *Achromobacter xylosoxidans* strain DEPA3

Culture 2: *Pseudomonas plecoglossicida* strain DEPB3

Culture 3: *Enterobacter cloacae* strain DEPC1

The whole soil sample from the pots was analyzed for the residual DEP at 10, 20 and 30 days incubation. The extraction was initiated with acetone where 30 mL acetone was added to the sample, mixed thoroughly by shaking for half an hour on mechanical shaker. After setting down of the soil, the supernatant was decanted on filter paper and collected in the conical flask. Again, the residue was treated with 15 mL acetone with 15 minutes shaking followed by decantation and filtration. Same procedure was followed using 5 mL acetone with manual shaking of 5 minutes. The collected supernatant was treated with 15% aq. NaCl and poured in a separating funnel, followed by addition of 50 mL DCM. The separating funnel was used for partition and collected the lower layer for further use. Same procedure was repeated using 30 mL followed by 10 mL DCM and lower layer was collected in each step. The whole collected sample was taken to rotary evaporimeter where DCM was evaporated and the residue was further treated with 2 mL methanol and filtered through 0.22 micron filter.

The final titrate was estimated using HPLC. The dried DEP extracted sample was dissolved in 2.0 mL methanol (HPLC grade) and filtered through cellulose nitrate membrane (0.45 μ m). 10 μ L sample was analyzed with a Waters 2487 HPLC equipped with a UV-VIS (Waters-2487) detector set at wavelength of 254 nm using reverse phase C-18 column (size 250 x 4.6 mm) with particle size 5 μ m was used to carry out separation. The isocratic mobile phase was methanol: water (80:20 v/v) and flow rate was 1 mL min⁻¹. The DEP concentrations were calculated on the basis of peak area measurements by comparison with an external standard of known concentration of DEP (300 ppm) prepared in methanol. Finally, residual DEP has calculated as similar degradation in PAEs and MS broth DEP.

3.17. HPLC analyses for DEHP degradation by selected isolates in MSM broth

3.17.1. Preparation of mother culture:

The selected isolates were inoculated in 50 mL MS broth containing 100 ppm DEHP and kept on an incubator shaker at 30.0 $\pm$ 2°C for 48 hrs. The bacterial count was enumerated using serial dilution technique for each isolates.

3.17.2. Preparation of DEHP stock solution:

Three grams DEHP was dissolved in 100 mL MS broth for preparing 30000 ppm stock solution (3g/100 mL). Transferred one mL DEHP from the stock solution in 100 mL MS broth in 250 mL conical flask. Each flask was inoculated with 5 mL bacterial isolates and incubated at 30°C in incubator shaker at 200 rpm. The samples were collected at 10, 20 and 30 days interval and analyzed further. The pH of medium was maintained at 7.0±0.2 and the bacterial count was $6.0 \times 10^5 \text{ mL}^{-1}$ - $2.87 \times 10^6 \text{ cfu/mL}$. Degradation study was carried out for each isolate singly as well as in combination with each other, in other words, consortium study was also carried out.

3.17.3. Estimation of Residual DEHP:

All the samples collected at 10, 20 and 30 days of incubation were analyzed for residual DEHP. Each flask containing 100 mL MS broth and 5 mL bacterial culture after the desired incubation was taken for further analysis. Added 50 mL 15 % aqueous NaCl followed by 50 mL DCM. The contents were poured in separating funnel and after thorough mixing, lower layer was collected after filtration through sodium sulfate so as excess of water is adsorbed. Same procedure was repeated using 30 mL and 10 mL DCM in separating funnel. DCM from the filtrate was evaporated using rotary evaporimeter.

Finally, the dried DEHP sample was dissolved in 2.0mL methanol (HPLC grade) and filtered through cellulose nitrate membrane (0.45μ). Ten μL sample was analyzed with Waters 2487 HPLC equipped with a UV-VIS (Waters-2487) detector set at wavelength of 254 nm using reverse phase C-18 column (size 250 x 4.6 mm) with particle size 5mm to carry out separation. The isocratic mobile phase was methanol: water (80:20 v/v) and flow rate were 1 mL min^{-1} . The DEHP concentration was calculated on the basis of peak area measurements by comparison with an external standard of known concentration of DEHP (300 ppm) prepared in methanol.

Finally, residual DEHP was calculated similarly like residual PAEs and the degradation was calculated as:

$$\% \text{ DEHP Degradation} = \frac{A_0 - A_n}{A_0} \times 100$$

where, A_n = Amount recovered ($\mu\text{g mL}^{-1}$ or $\mu\text{g g}^{-1}$) on n days; A_0 = Amount recovered ($\mu\text{g mL}^{-1}$ or $\mu\text{g g}^{-1}$) on 0 day; n= Time interval after fortification (i.e. 10, 20, 30.....days)

3.18. Degradation of DEHP in microcosm

Soil with no history of DEHP accumulation was collected from IARI farm. The collected soil was sandy loam in texture having 0.52% total organic carbon, 58.06 kg ha⁻¹ available N, 10.38 kg ha⁻¹ available P, and 105.25 kg ha⁻¹ exchangeable K. The soil was passed through 0.2 mm sieve. The sterile soil was fortified with analytical grade DEHP (SIGMA-ALDRICH Inc) under aseptic conditions to obtain final concentration of 300 ppm (300 mg Kg⁻¹) in each pot.

Each pot was filled with 100 gm fortified soil at 30 mg DEHP/100 gm soil. Sterile water was added in each pot for moistening the soil. The mother culture of each isolate was prepared in 50 mL MS broth containing 100 ppm DEHP. 5 mL bacterial culture was transferred singly as well as in combination to each pot and incubated for 10, 20 and 30 days interval.

Following treatments were taken with three replications each:

T1: Culture 1 + soil with 300 ppm of DEHP

T2: Culture 2 + soil with 300 ppm of DEHP

T3: Culture 3 + soil with 300 ppm of DEHP

T4: Culture 1 + 2 + 3 + soil with 300 ppm of DEHP

T5: Un-inoculated control.

Whereas, Culture 1: *Pseudomonas* sp. DEHPA2

Culture 2: *Pseudomonas* sp. DEHPB2

Culture 3: *Pseudomonas* sp. DEHPC2

The whole soil sample from the pots was analyzed for the residual DEHP at 10, 20 and 30 days incubation. The extraction was initiated with acetone and 30 mL acetone was added to the sample, mixed thoroughly by shaking for half an hour on mechanical shaker. After the settling down of the soil, the supernatant was decanted on filter paper and collected in the conical flask. Again, the residue was treated with 15 mL acetone with 15 minutes shaking followed by decantation and filtration. Same procedure was followed using 5 mL acetone with manual shaking of 5 minutes. The collected supernatant was treated with 15% aq. NaCl and poured in a separating funnel, followed by addition of 50 ml DCM. The separating funnel was used for partition and collected the lower layer for further use. Same procedure was repeated

using 30 ml followed by 10 mL DCM and lower layer was collected in each steps. The whole collected sample was taken to rotary evaporimeter where DCM was evaporated and the residue was further treated with 2 mL methanol and filtered through 0.22 micron filter.

The final titrate was estimated using HPLC. The dried DEHP extracted sample was dissolved in 2.0 mL methanol (HPLC grade) and filtered through cellulose nitrate membrane (0.45 μm). 10 μL sample was analyzed with a Waters 2487 HPLC equipped with a UV-VIS (Waters-2487) detector set at wavelength of 254 nm using reverse phase C-18 column (size 250 x 4.6 mm) with particle size 5mm was used to carry out separation. The isocratic mobile phase was methanol: water (80:20 v/v) and flow rate were 1 mL min^{-1} . The DEHP concentrations were calculated on the basis of peak area measurements by comparison with an external standard of known concentration of DEHP (300 ppm) prepared in methanol. Finally, residual DEHP was calculated as similar as degradation in PAEs and DEP.

3.19. Data analysis

Statistical analyses of the data were performed using STATISTICA 10. Analysis of variance and separation of means by least significant differences were performed by using the general linear models (GLM). Unless indicated otherwise, differences were considered only when significant at $P = 0.05$.

4. RESULTS

4.1. Physico-chemical characteristics of soil samples from the phthalate contaminated sites

The soil samples were collected from three different sites namely inside the greenhouse, plastic mulching site and outside the greenhouse at Centre for Cultivated Protection Technology (CPCT) IARI, New Delhi, India as it was found to be contaminated with phthalate esters. The soil samples were labelled as DK1 (Inside green house), DK 2 (Mulching site) and DK 3 (Outside green house). All the collected samples were analysed for physicochemical parameters (Table: 4.1). On the whole, the soil was sandy loam in texture and samples showed marginal differences in pH, Electrical conductivity (EC), organic carbon (OC), P_2O_5 , K_2O , sulphur, boron, iron, manganese, and zinc content.

The pH range of three collected samples was observed as 7.1-7.65 showing neutral to slightly alkaline nature where the soil sample DK 1(Inside green house) showed maximum pH 7.65 followed by DK 3 (pH 7.4) and DK 2 (pH 7.1) respectively.

EC of the soil samples was observed in the range 0.457-0.496 dS m^{-1} , and the organic carbon content was very low in all the three samples. The organic carbon percentage range was observed from 0.1 -0.6% where DK 2 showed maximum organic carbon (0.6%) followed by DK 1 (0.2%) and minimum in DK 3(0.1%).

The extractable potassium was observed within 486.5 kg ha^{-1} to the maximum 626.2 kg ha^{-1} where DK 3 (mulching site) showed highest K_2O content followed by DK 2 (560.4 kg ha^{-1}) and lowest in DK 1 (486.5 kg ha^{-1}) while phosphorus content (P_2O_5) was obtained in the range of 15.53 kg ha^{-1} to 19.2 kg ha^{-1} and the soil sample DK 1 (19.2 kg ha^{-1}) showed highest P_2O_5 content followed by DK 2 (18.6 kg ha^{-1}) and DK 3 (15.53 kg ha^{-1}) respectively.

The presence of Zn was found maximum in sample DK 2 as compared to DK 1 and DK 3 and the zinc range was observed as 0.5 -18.8 kg ha^{-1} while sulfur, boron, iron, and manganese were present as 4.84 -5.41 $\mu g\ kg^{-1}$, 0.416 to 2.12 $\mu g\ kg^{-1}$, 0.63-16.74 $\mu g\ kg^{-1}$ and 3.30 to 5.41 $\mu g\ kg^{-1}$ soil respectively.

Among the various nutrients sulphur content was observed in the range of 24.8-50.4 $\mu\text{g kg}^{-1}$ where the maximum sulphur content was observed in the soil sample DK 2 (50.4 $\mu\text{g kg}^{-1}$).

Boron content was also found maximum in sample DK 2 (2.125 $\mu\text{g kg}^{-1}$) followed by DK 3 and DK 2 and iron content was observed highest in DK 3 (16.6 $\mu\text{g kg}^{-1}$) and the range of iron was as observed was 0.06 -16.6 $\mu\text{g kg}^{-1}$ while manganese content was maximum in soil sample labelled as DK 2 (54.1 $\mu\text{g kg}^{-1}$) followed by DK 1 (33.3 $\mu\text{g kg}^{-1}$) and DK 3 (26.3 $\mu\text{g kg}^{-1}$).

All the observations showed variation in different nutrients content in three soil samples DK1, DK 2 and DK 3 but most of nutrients were found in high concentration in sample DK 2.

4.2. Fortification of soil to recover DEP and DEHP

To optimize and form a standard protocol for the estimation of residual phthalate esters mainly DEP and DEHP from soil and MS broth, an experiment was carried out where both soil and MS broth fortified with three different concentrations mainly 10 ppm, 100 ppm and 300 ppm DEP and DEHP respectively. Results obtained in (Table 4.2) revealed that when DEP concentration was increased in broth from 10 ppm to 300 ppm the recovery of same compound was found in range 94.8 to 95.3% respectively. It was further observed that an increase of DEP concentration from 100 ppm to 300 ppm showed no effect or change on recovery resulting 95.3% recovery at 100 ppm as well as 300 ppm. In case of soil, there was an increase in recovery of DEP from 10 ppm to 300 ppm showing 91.6 -91.96% recovery respectively.

Similarly, the other important compound DEHP was also studied for recovery in MS broth and soil. The DEHP recovery increased from 90.93 to 91.33% along with increase in DEHP concentration from 10 ppm to 300 ppm in MS broth and it also increased in soil showing 93.4 to 93.76% recovery (Figure 7 & 8).

4.3. Isolation of PAEs degrading microorganisms

Enrichment technique was used for the isolation of PAEs degrading bacteria from the three soil samples collected from different sites at Centre for Cultivated Protection Technology (CPCT) IARI, New Delhi, India (Plate 2 & 3).

Table 4.1: Physicochemical characteristics of soil samples from the phthalate contaminated sites of CPCT IARI, New Delhi

Parameters	DK 1 (Inside green house)	DK 2 (Mulching site)	DK 3 (Outside green house)	CV	LSD (P<0.05)
	Mean± SD				
OC (%)	0.213±0.011	0.609±0.0060	0.170±0.001	2.446	0.041
EC (ds m ⁻¹)	0.457±0.003	0.495±0.004	0.0496±0.005	2.446	0.0072
pH	7.650±0.030	7.147±0.042	7.407±0.051	2.446	0.0837
P ₂ O ₅ (kg ha ⁻¹)	19.200±0.767	18.667±4.081	15.533±0.666	2.446	4.850
K ₂ O(kg ha ⁻¹)	486.533±4.576	560.400±6.920	626.267±5.853	2.446	11.711
Zn(µg kg ⁻¹)	0.500±0.100	8.863±0.335	4.300±0.200	2.446	0.465
S(µg kg ⁻¹)	24.840±0.075	50.413±0.974	40.673±0.885	2.446	1.520
B(µg kg ⁻¹)	0.416±0.011	2.125±0.032	0.531±0.008	2.446	0.04
Fe(µg kg ⁻¹)	0.063±0.015	15.747±1.374	16.643±1.061	2.446	2.002
Mn(µg kg ⁻¹)	33.307±0.110	54.173±2.668	26.323±1.153	2.446	3.355

Table 4.2: Recovery of DEP and DEHP using soil fortification

Fortification ($\mu\text{g mL}^{-1}$)	Recovery (%)			
	DEP (Broth)	DEP(Soil)	DEHP (Broth)	DEHP (Soil)
	Mean $\pm$ SD			
10 ppm	94.8 $\pm$ 0.242	91.6 $\pm$ 0.209	90.93 $\pm$ 0.404	93.4 $\pm$ 0.312
100 ppm	95.33 $\pm$ 0.196	91.83 $\pm$ 0.312	91.6 $\pm$ 0.311	93.73 $\pm$ 0.242
300 ppm	95.3 $\pm$ 0.211	91.96 $\pm$ 0.231	91.33 $\pm$ 0.319	93.76 $\pm$ 0.311

Table 4.3: Morphological characterization selected for DEP degradation

Strain Number	Colony Colour	Size and Pigmentation	Margin	Elevation	Shape
DEP A1	Shiny blackish	Punctiform pigmented	Entire	Flat	Rods
DEP A2	Creamy white	Circular non pigmented	Undulate	Convex	Cocci
DEP A3	Off white	Punctiform non pigmented	Lobate	Raised	Rods
DEP B1	Off white	Punctiform non pigmented	Entire	Flat	Rods
DEP B2	Shiny whitish	Circular pigmented	Lobate	Flat	Rods
DEP B3	Creamy white	Irregular pigmented	Curled	Raised	Rods
DEP C1	Creamy white	Circular pigmented	Undulate	Flat	Rods
DEP C2	Dull	Circular pigmented	Lobate	Umbonate	Rods
DEPC3	White	Punctiform non pigmented	Undulate	Umbonate	Rods

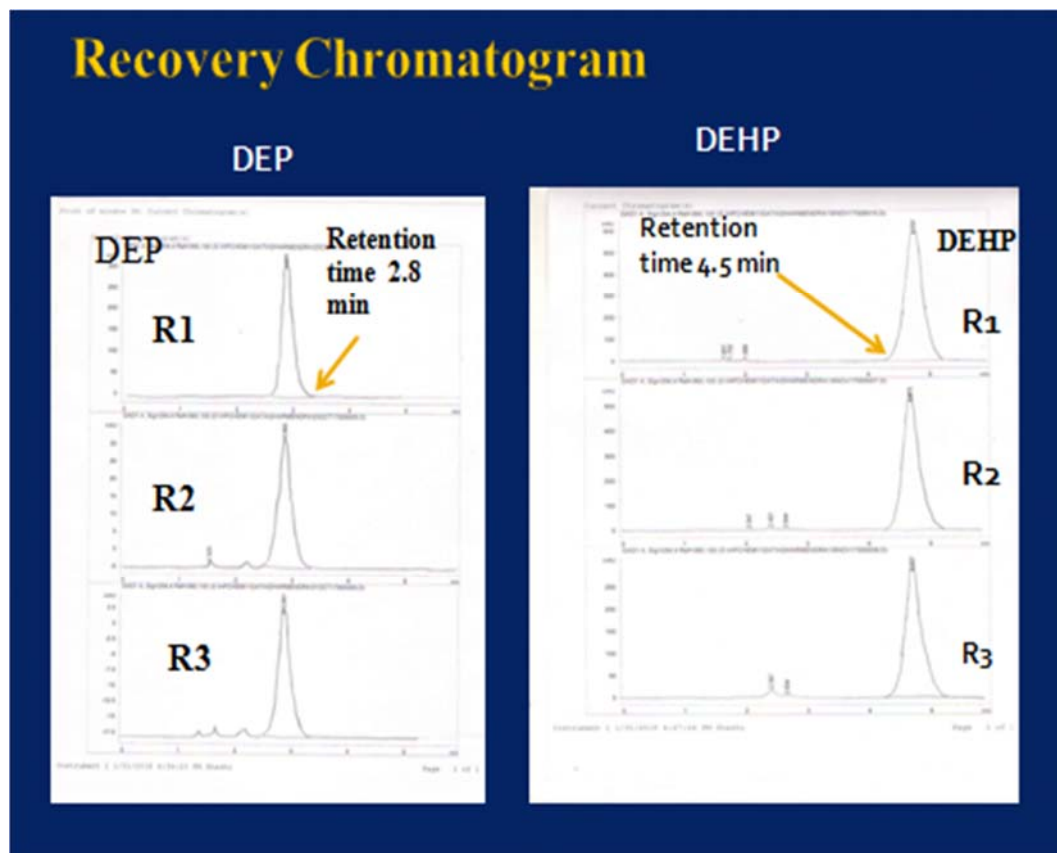


Figure 7: Recovery chromatogram of DEP and DEHP

Standard Chromatogram of PAEs

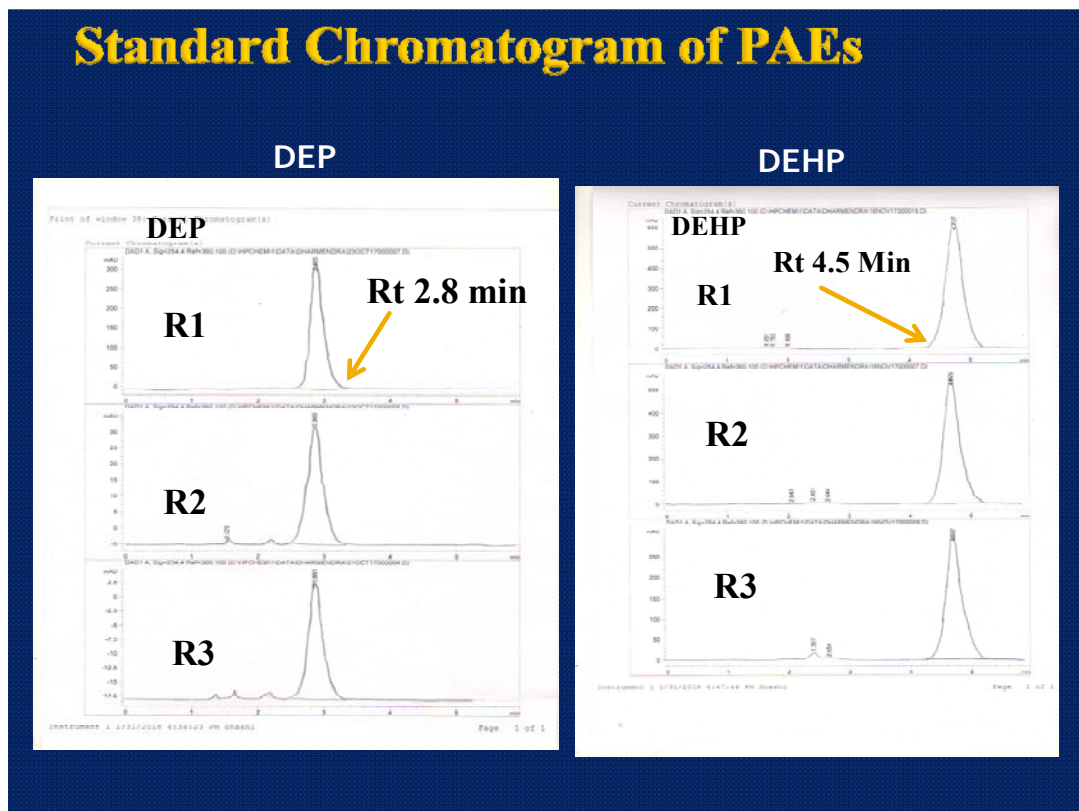


Figure 8: Standard chromatogram of DEP and DEHP

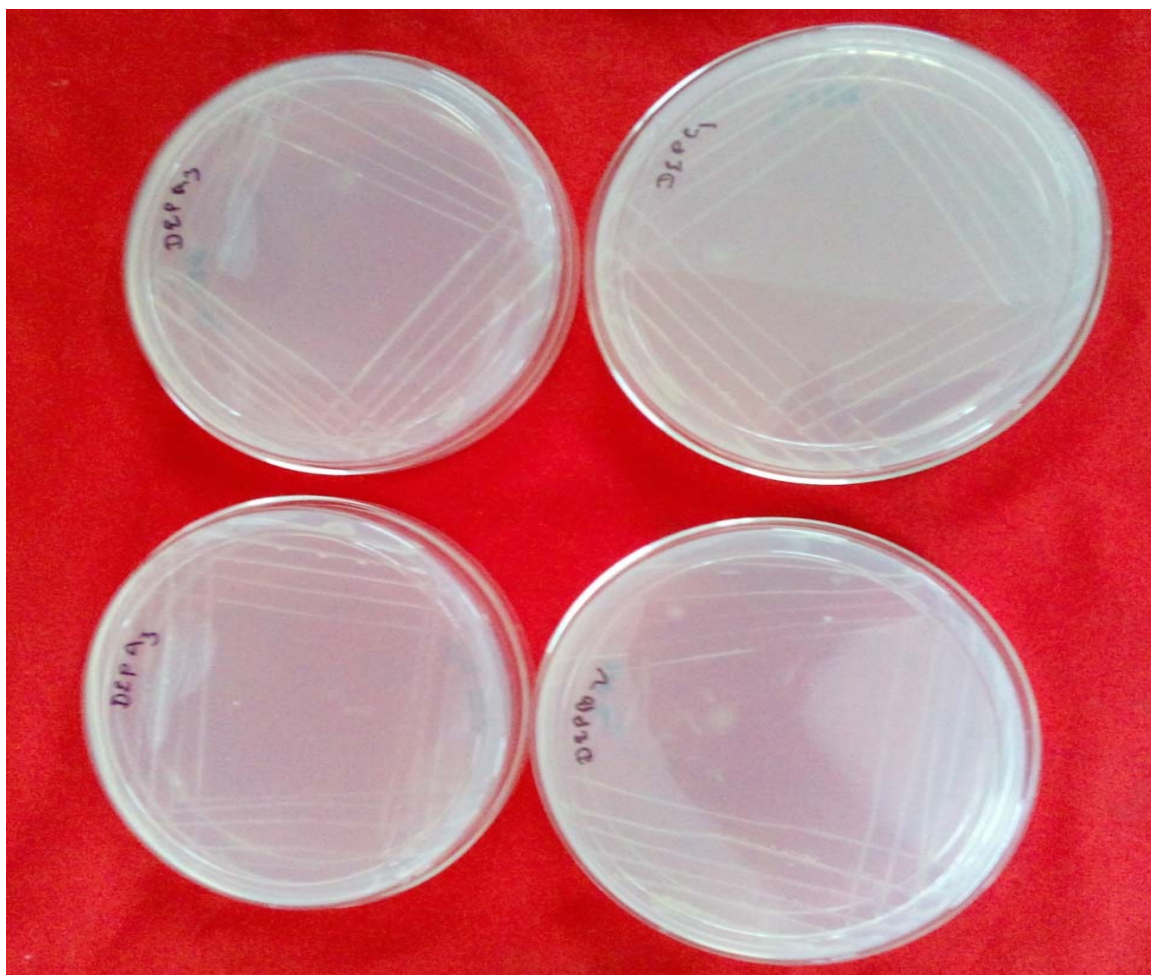


Plate 2: Different isolates selected for PAEs degradation

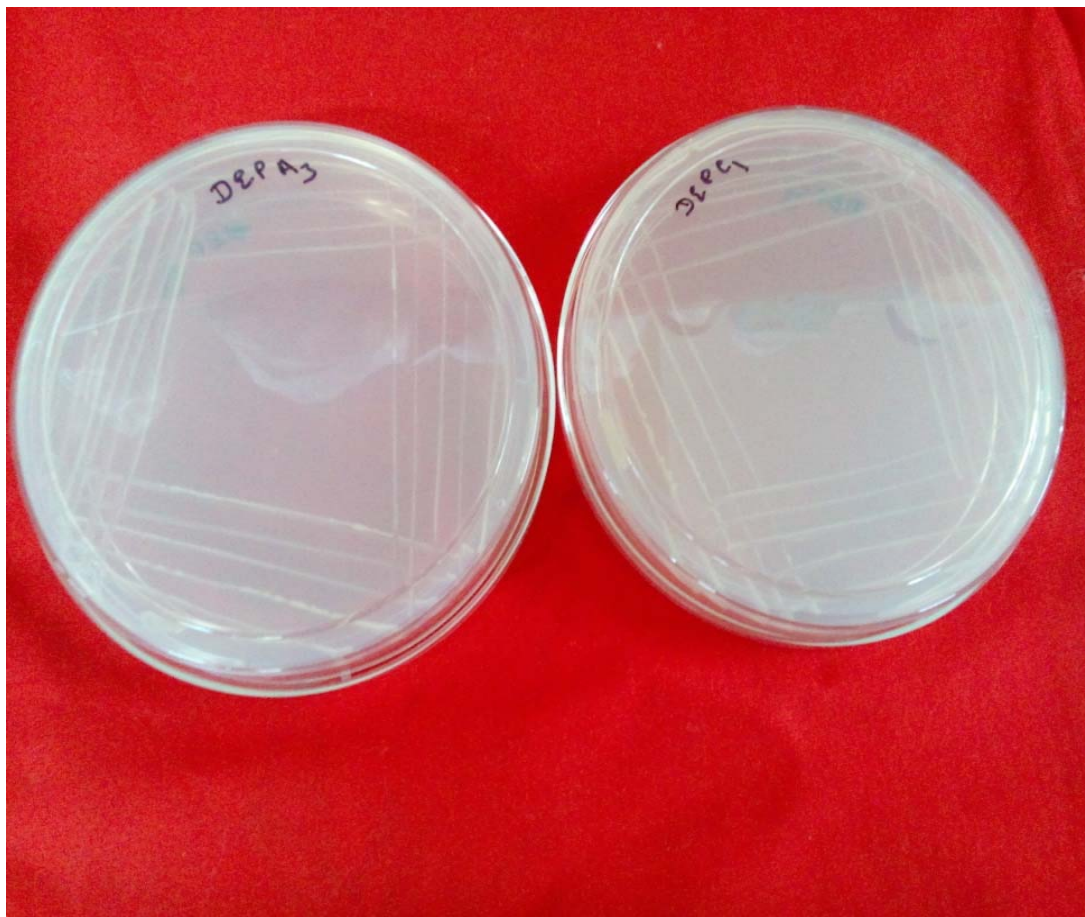


Plate 3: Efficient bacterial isolates for DEP degradation

4.3.1. Morphological characterization of DEP and DEHP degrading bacterial isolates

The Mineral salt medium (MSM) containing 50 ppm DEP and DEHP as sole carbon source was used for bacteria and a total ninety bacterial isolates were selected on visual basis which was further screened for morphological characterization like pigmentation, colour, margin, shape, size and on this basis, nine bacterial isolates designated as DEP A1, DEP A2, DEP A3, DEP B1, DEP B2, DEP B3, DEP C1, DEP C2, DEP C3 were selected as DEP degrading isolates while DEHP A1, DEHP A2, DEHP A3, DEHP B1, DEHP B2, DEHP B3, DEHP C1, DEHP C2, DEHP C3 were selected for DEHP degradation (Table 4.3 & 4.4).

All the 90 isolates were purified to single colony for morphological characterization and 18 isolates were selected for further studies (Table 4.3 & 4.4). All the isolates were found to be rod-shaped except DEP B2 which was cocci. Most of the bacterial colonies were whitish in colour, however, variation in white colour was observed from dull, creamy white to off-white while two colonies were shiny with blackish shade. Margins of colonies varied from circular, irregular to punctiform with entire, curled and undulated margin. Some of colonies were pigmented. The margins of colonies were either flat or raised. These bacteria were designated as DEP A1 to DEP A15, DEP B1 to DEP B15, DEP C1 to DEP C15 for DEP degradation, DEHP A1 to DEHP A15, DEHP B1 to DEHP B15 and DEHP C1 to DEHP C15 for DEHP degradation (Table 4.3 & 4.4). Hence, a total of 18 bacterial isolates based on morphology were finally selected for further studies.

4.3.2. Biochemical characterization of DEP and DEHP degrading bacterial isolates

The nine DEP degrading bacterial isolates were further enzymatically characterized where all the nine isolates were found to produce cytochrome oxidase, degrade H_2O_2 by producing enzyme catalase and able to hydrolyse esters with the help of esterase enzyme but out of nine isolates four isolates namely viz. DEP A1, DEP A2, DEP A3, and DEP B1 were unable to produce urease enzyme for hydrolysis of urea and were able to produce amylase for starch hydrolysis. DEP B2, DEP B3, DEP C1 and DEP C1 except DEP C2 and DEP C3 all bacterial isolates showed capability to

hydrolyse starch. All the nine isolates were found to be Gram-negative rods except DEP A2 which was found to be cocci (Table 4.5 & 4.6).

Similarly, the nine bacterial isolates for DEHP degradation were also characterized enzymatically and all nine were found to produce cytochrome oxidase, catalase, and esterase but variation was again observed for urea and starch hydrolysis. DEHP A1, DEHP A2, DEHP B1, DEHP B2, DEHP B3, and DEHP C2 were unable to hydrolyse urea and DEHP B3, DEHP C1 and DEHP C2 were not able to hydrolyse starch (Table 4.5 & 4.6). All the isolates were found to be gram-negative rods.

4.4. Growth of bacterial isolates at different concentrations of DEP and DEHP:

The selected isolates for DEP and DEHP degradation were further screened for their growth in respective MS broth. Nine DEP and nine DEHP bacterial isolates were grown on different concentration from 100 to 700 ppm of respective compound in mineral salt medium broth. Growth was measured in term of protein in μg per mL.

Results obtained in (Table 4.7) revealed that all the nine isolates were able to grow in MS broth with different concentration of DEHP with variation in growth. The isolates DEHP A1 showed an increase in protein concentration with an increase of DEHP till 500 ppm but at 700 ppm although growth was very poor and the protein concentration varied from 48.00 to 68.3 ppm and then drastically lowered to 12.00 ppm whereas DEHP A2, DEHP A3, and DEHP B1 showed a decline in protein concentration from 67.00 to 4.00 ppm with an increase in DEHP from 100 ppm to 700 ppm. Further, it was observed that isolates DEHP C1 and DEHP C2 showed first an increase in protein concentration till 300 ppm DEHP and later declined with increase in DEHP concentration. On the basis of protein concentration, three isolates DEHP A1, DEHPB2, and DEHP C2 were further selected for molecular characterization.

Similarly, the nine isolates for DEP degradation also showed variation in protein content and almost five isolates DEP A1, DEP A2, DEP B2, DEP B3, and DEP C3 were able to grow at 300 ppm DEP while other four isolates showed good growth at 100 ppm DEP, moderate at 300 ppm, low at 500 ppm and poor at 700 ppm. Hence the isolates DEP A2, DEP B2, and DEP C3 as DEP degraders were selected for final molecular characterization (Table 4.8).

Table 4.4: Morphological characterization of DEHP degrading selected bacterial isolates

Strain Number	Colony Colour	Size and Pigmentation	Margin	Elevation	Shape
DEHP A1	Creamy white	Punctiform non pigmented	Undulate	Raised	Rods
DEHP A2	White off	Irregular non pigmented	Entire	Pollinated	Rods
DEHP A3	Dull mucoid	Irregular non pigmented	Curled	Raised	Rods
DEHP B1	Creamy white	Circular non pigmented	Entire	Raised	Rods
DEHP B2	Dull	Irregular pigmented	Undulate	Lobat	Rods
DEHP B3	Shiny	Punctiform pigmented	Curled	Raised	Rods
DEHP C1	Creamy white	Irregular non pigmented	Entire	Pollinated	Rods
DEHP C2	Shiny	Irregular non pigmented	Lobate	Raised	Rods
DEHP C3	Creamy white	Circular non pigmented	Entire	Convex	Rods

Table 4.5: Biochemical characterization of DEP degrading selected bacterial isolates

Strain Number	Gram reaction	Oxidase	Catalase	Urease	Starch hydrolysis	Esterase hydrolase
DEP A1	-ve	+	+	-	+	+
DEP A2	-ve	+	+	-	+	+
DEP A3	-ve	+	+	-	+	+
DEP B1	-ve	+	+	+	+	+
DEP B2	-ve	+	+	-	+	+
DEP B3	-ve	+	+	-	+	+
DEP C1	-ve	+	+	-	+	+
DEP C2	-ve	+	+	-	-	+
DEPC3	-ve	+	+	+	-	+

Table 4.6: Biochemical characterization of DEHP degrading selected bacterial isolates

Strain Number	Gram reaction	Oxidase	Catalase	Urease	Starch hydrolysis	Esterase hydrolase
DEHP A1	-ve	+	+	+	+	+
DEHP A2	-ve	+	+	-	+	+
DEHPA3	-ve	+	+	+	+	+
DEHP B1	-ve	+	+	-	+	+
DEHP B2	-ve	+	+	-	+	+
DEHP B3	-ve	+	+	-	-	+
DEHP C1	-ve	+	+	+	-	+
DEHP C2	-ve	+	+	-	-	+
DEHP C3	-ve	+	+	+	+	+

Table 4.7: Total Protein ($\mu\text{g mL}^{-1}$) content of selected bacterial isolates at different concentration of DEHP

Isolate no.	Protein concentration (ppm)			
	100	300	500	700
DEHP A1	48.000 ± 0.577	50.000 ± 1.155	68.333 ± 1.202	12.000 ± 1.155
DEHP A2	67.000 ± 1.155	37.000 ± 0.577	13.333 ± 0.667	4.000 ± 0.557
DEHP A3	52.667 ± 0.882	20.000 ± 1.155	14.333 ± 0.882	6.000 ± 0.577
DEHP B1	22.000 ± 1.528	39.667 ± 0.882	24.000 ± 0.557	4.000 ± 0.577
DEHP B2	41.333 ± 0.882	68.000 ± 0.557	49.667 ± 0.882	11.667 ± 0.882
DEHP B3	50.333 ± 0.882	67.333 ± 0.882	18.333 ± 0.882	6.333 ± 0.882
DEHP C1	38.667 ± 0.882	17.667 ± 0.882	40.333 ± 0.667	14.333 ± 1.028
DEHP C2	22.333 ± 1.202	31.000 ± 1.155	18.667 ± 0.882	5.000 ± 0.882
DEHP C3	38.333 ± 0.882	80.333 ± 0.882	54.000 ± 0.577	9.667 ± 0.577
C.D.	3.049	2.783	2.467	3.425
SE(m)	1.018	0.930	0.824	1.144
SE(d)	1.440	1.315	1.165	1.618
C.V.	4.283	3.286	3.156	5.307

Table 4.8: Total Protein ($\mu\text{g mL}^{-1}$) content of bacterial at different concentration of DEP

Isolate no.	Protein concentration (ppm)			
	100	300	500	700
DEP A1	59.667 ± 0.882	55.667 ± 1.202	28.333 ± 1.202	12.000 ± 1.155
DEP A2	68.333 ± 0.882	49.667 ± 0.882	12.333 ± 0.882	9.000 ± 0.557
DEP A3	50.333 ± 0.882	19.000 ± 0.882	33.333 ± 0.882	6.000 ± 0.577
DEP B1	15.000 ± 0.577	40.667 ± 0.882	43.000 ± 0.882	6.000 ± 0.577
DEP B2	49.667 ± 1.453	68.667 ± 0.667	36.667 ± 1.764	3.667 ± 0.882
DEP B3	79.667 ± 1.453	52.667 ± 0.882	12.333 ± 1.000	6.333 ± 0.882
DEP C1	71.000 ± 0.577	43.667 ± 0.882	27.333 ± 0.882	14.333 ± 1.028
DEP C2	22.333 ± 0.882	41.333 ± 1.155	17.667 ± 0.577	5.000 ± 0.882
DEP C3	38.333 ± 0.882	58.000 ± 0.577	54.000 ± 0.822	9.667 ± 1.115
C.D.	2.957	3.720	3.121	2.976
SE(m)	0.988	1.242	1.042	0.994
SE(d)	1.397	1.757	1.474	1.405
C.V.	3.462	4.970	4.526	4.495

4.5. Molecular characterization of bacterial isolates using 16s rRNA and phylogenetic analysis:

The final eleven selected bacterial isolates were further studied for molecular characterization. The genomic DNA of all eleven bacterial isolates was extracted and 16S rRNA gene was amplified using both forward and reverse primer (pA and pH, respectively). The reverse and forward purified 16S rRNA gene from all the isolates was further sequenced. After obtaining the forward and reverse sequences of 16S rRNA gene (approx. 600-700 bp), contigs were made using online software CAP3. All the sequences were compared with 16S rRNA gene sequences available in the Gene Bank data bases by BLASTn search and the identity of isolates is given in (Table 4.9).

The partial sequences of 16S rRNA gene sequences after analysis were submitted to NCBI Gene Bank database under accession numbers MF661913 to MF661913 (Table 4.9). Multiple sequence alignment of approx 1500-bp sequences was performed using CLUSTALW, version 1.8 and phylogenetic tree was constructed (Figure 9).

The phylogenetic relationships of the isolates as inferred from comparison of partial sequences (approx 1500 bp) of the 16S rRNA genes showed that these isolates fell into two major lineages of domain bacteria; the γ -*Proteobacteria* and β -*Proteobacteria*. The ten isolates of γ -*Proteobacteria*, matched with sequences of *Pseudomonas aeruginosa* (DEHPA1, DEHPB1, and DEHPC3), *Pseudomonas sp.* (DEHPA2, DEHPB2, and DEHPC2), *Pseudomonas plecoglossicida* (DEPB3) and *Enterobacter sp.* (DEPA1 and DEPC1). The one isolate of β -*Proteobacteria*, matched with sequences of *Achromobacter* (DEPA3) (Plate 4 & 5).

4.6. Microbial degradation of DEHP in MS broth

Compatibility: All the identified strains for DEP and DHEP were found compatible with each other as no antagonistic activity was observed on plates (Figure 10).

The three isolates viz *Pseudomonas sp.* DEHPA2, *Pseudomonas sp.* DEHPB2 and *Pseudomonas sp.* DEHPC2 were inoculated individually and also as mixed inoculum in equal ratio (1:1:1) of three together as consortium in MS broth containing 300 mg L⁻¹ DEHP as sole carbon source. All the flasks were inoculated for 30 days and flasks were removed in batches after every ten days interval and DEHP was

estimated in each sample. Results indicated that all the three bacteria were able to grow and utilize DEHP as energy source. There was reduction in pH where bacterial culture was inoculated in MS broth from 6.1 to 6.3 in all the flasks as compared to un-inoculated flask (Plate 6).

Pseudomonas sp. DEHPA2 showed a good amount of DEHP degradation in 30 days where the residual DEHP was found to be 72.7 ppm, 58.0 ppm and 43.3 ppm from initial 287 ppm DEHP after 10, 20 and 30 days of incubation respectively. Another bacterial isolate *Pseudomonas sp.* DEHPB2 was the most efficient DEHP degrader as compared to *Pseudomonas sp.* DEHPA2 and *Pseudomonas sp.* DEHPC2. These bacteria showed 70.0 ppm, 54.9 ppm, and 40.7 ppm DEHP after 10, 20 and 30 days respectively from initial 286 ppm DEHP. The bacterial strain *Pseudomonas sp.* DEHPC2 was also able to degrade DEHP from 286 ppm to 43.7 ppm after 30 days (Table 4.10, 4.11 & 4.12).

The most efficient degradation was observed when the three bacteria were inoculated as consortium and a rapid decline was observed from 285 ppm to 49.1 ppm, 32.4 ppm and 11 ppm DEHP after 10, 20 and 30 days of incubation. The percentage degradation of DEHP in MS broth (Table 4.13) varied significantly from 10 days to 30 days after inoculation. The percentage DEHP degradation was observed as 79.8 - 87.8% after 10 days, 87.1 - 91.3% after 20 days and 88.7 to 96.6% after 30 days of incubation.

The results highlighted the role of selected bacterial isolates for degradation of DEHP in MS broth. The maximum degradation within 30 days was observed with mixed culture as compared to individual bacterial isolates.

4.7.Evaluation of selected bacteria for DEHP degradation in soil microcosm

After MS broth study, the three selected bacteria *Pseudomonas sp.* DEHPA2, *Pseudomonas sp.* DEHPB2 and *Pseudomonas sp.* DEHPC2 were inoculated in soil for degradation of DEHP. All the bacterial isolates were inoculated individually as well as a consortium (1:1:1) in 50 gm soil containing 300 ppm DEHP. An un-inoculated control was also set from day of start of experiment.

The results obtained in Table 4.14, 4.15 & 4.16 shows that in microcosm also the inoculated bacterial cultures degraded DEHP efficiently where *Pseudomonas sp.* DEHPA2 was able to degrade DEHP resulting in 51.8 ppm as residual from initial 275

Table 4.9: Molecular characterization of all selected bacterial isolates using 16s rRNA

S.No.	Isolates	Scientific names	Accession numbers	Similarity
1.	DEPA1	<i>Enterobacter cloacae</i> strain DEPA1	MF661913	98 %
2.	DEPA3	<i>Achromobacter xylosoxidans</i> strain DEPA3	MF661914	97 %
3.	DEPB3	<i>Pseudomonas plecoglossicida</i> strain DEPB3	MF661915	99 %
4.	DEPC1	<i>Enterobacter cloacae</i> strain DEPC1	MF661916	99 %
5.	DEHPA1	<i>Pseudomonas aeruginosa</i> strain DEHPA1	MF661917	98 %
6.	DEHPA2	<i>Pseudomonas</i> sp. DEHPA2	MF661918	97 %
7.	DEHPB1	<i>Pseudomonas aeruginosa</i> strain DEHPB1	MF661919	99 %
8.	DEHPB2	<i>Pseudomonas</i> sp. strain DEHPB2	MF6619320	98 %
9.	DEHPC1	<i>Pseudomonas aeruginosa</i> strain DEHPC1	MF661921	97 %
10.	DEHPC2	<i>Pseudomonas</i> sp. strain DEHPC2	MF661922	99 %
11.	DEHPC3	<i>Pseudomonas aeruginosa</i> strain DEHPC3	MF661923	97 %

Table 4.10: Effect of bacterial inoculation on DEHP degradation in MS broth

S.No	Isolates	Days of incubation (ppm)			
		0 d	10 d	20 d	30 d
1.	<i>Pseudomonas</i> <i>sp.</i> DEHPA2	287	72.7	58	43.3
2.	<i>Pseudomonas</i> <i>sp.</i> DEHPB2	286	70.5	54.9	40.7
3.	<i>Pseudomonas</i> <i>sp.</i> DEHPC2	286	71.6	55.9	43.7
4.	1+2+3	285	49.1	32.4	11

Table 4.11: A X B X C Mean Table of 4.10

	A₁		A₂		A₃		A₄	
	B₁	B₂	B₁	B₂	B₁	B₂	B₁	B₂
C₁	286.700	286.700	286.333	286.333	286.467	286.467	285.333	285.333
C₂	286.333	72.700	286.167	70.500	286.500	71.633	286.667	49.067
C₃	286.267	58.033	286.367	54.933	286.533	55.933	286.000	32.400
C₄	285.700	43.300	286.000	40.700	286.267	43.733	285.267	11.000

Where

A₁= Isolates DEHPA2, A₂= Isolates DEHPB2, A₃= Isolates DEHPC2, A₄= Isolates A+B+C

B₁= Control, B₂=Inoculate, C₁= 0 day, C₂= 10 days, C₃= 20 days, C₄= 30 days

Table 4.12: SEM, SED and C.D. Table of 4.11

Factors	C.D.	SE(d)	SE(m)
Factor(A)	0.381	0.191	0.135
Factor(B)	0.269	0.135	0.095
Intrraction A X B	0.539	0.270	0.191
Factor(C)	0.381	0.191	0.135
Intrraction A X C	0.762	0.381	0.270
Intrraction B X C	0.539	0.270	0.191
Intrraction A X B X C	1.078	0.539	0.381

Table 4.13: Efficiency of DEHP degrading bacteria in MS broth

S.N.	Isolates	% DEHP degradation in MS broth		
		10 d	20 d	30 d
1	<i>Pseudomonas sp.</i> DEHPA2	79.85	88.22	90.21
2	<i>Pseudomonas sp.</i> DEHPB2	80.39	87.90	88.75
3	<i>Pseudomonas sp.</i> DEHPC2	82.02	87.12	92.44
4	1+2+3	87.86	91.38	96.61

Table 4.14: Evaluation of selected bacteria for DEHP degradation in soil microcosm

S.No	Isolates	Days of incubation (ppm)			
		0 d	10 d	20 d	30 d
1.	<i>Pseudomonas sp.</i> DEHPA2	275	92.8	66.3	51.8
2.	<i>Pseudomonas sp.</i> DEHPB2	276	90.2	64.1	49
3.	<i>Pseudomonas sp.</i> DEHPC2	275	93.9	67.6	50.2
4.	1+2+3	276	67.8	54.4	21

Table 4.15: A X B X C Mean Table of 4.14

	A₁		A₂		A₃		A₄	
	B₁	B₂	B₁	B₂	B₁	B₂	B₁	B₂
C₁	275.033	275.033	274.833	274.833	275.367	275.367	275.500	275.500
C₂	275.000	92.833	274.533	90.200	275.100	93.867	274.667	67.833
C₃	274.767	66.267	274.500	63.100	274.600	67.600	273.600	54.400
C₄	274.767	51.933	274.867	49.933	274.200	50.167	273.333	21.000

Table 4.16: SEM, SED and C.D. Table of 4.15

Factors	C.D.	SE(d)	SE(m)
Factor(A)	0.747	0.374	0.264
Factor(B)	0.528	0.264	0.187
Intraction A X B	1.056	0.529	0.374
Factor(C)	0.747	0.374	0.264
Intraction A X C	1.494	0.748	0.529
Intraction B X C	1.056	0.529	0.374
Intraction A X B X C	2.113	1.057	0.748

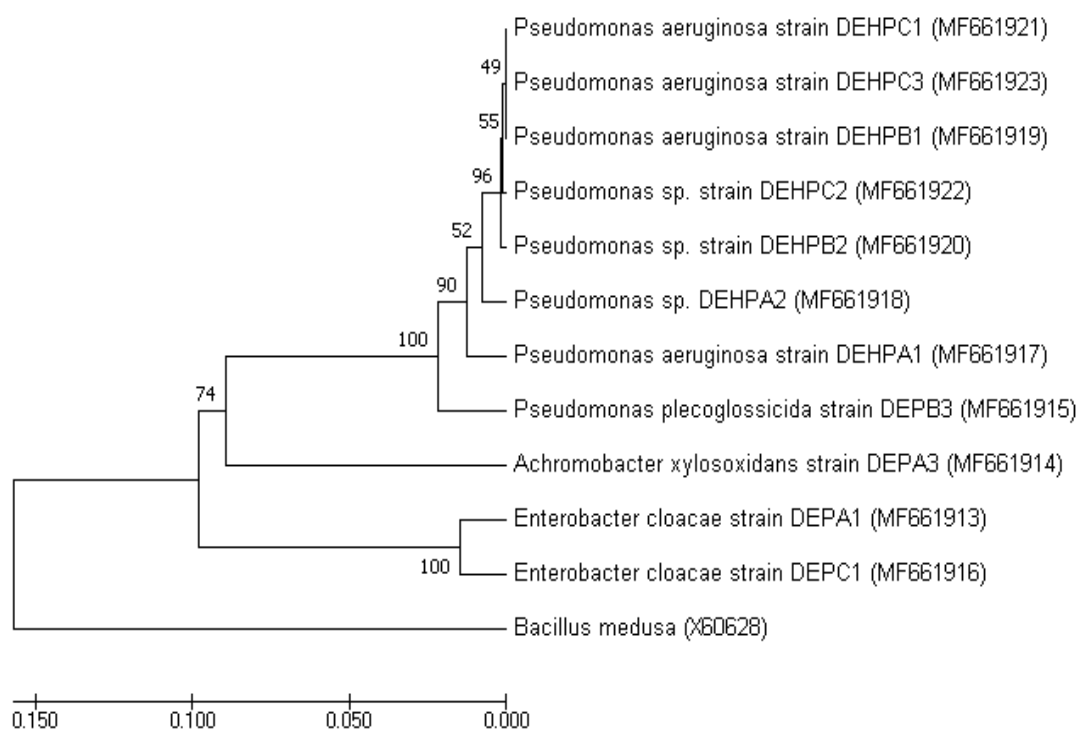


Figure 9: Phylogenetic tree of isolated screened and identified PAEs degraders

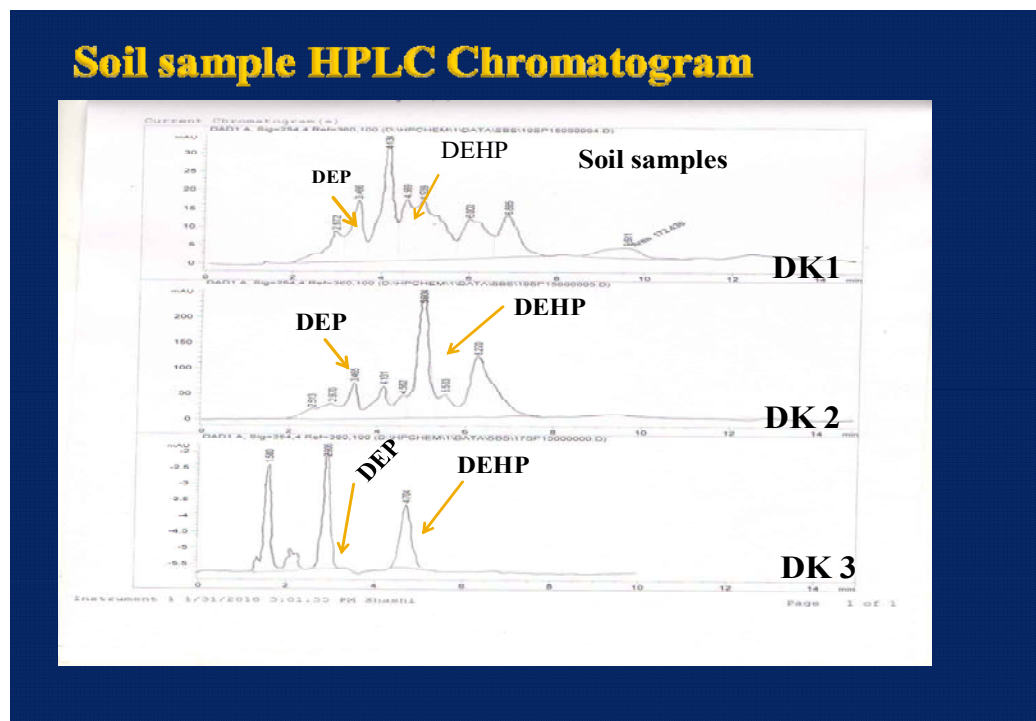


Figure 10: PAEs in soil sample

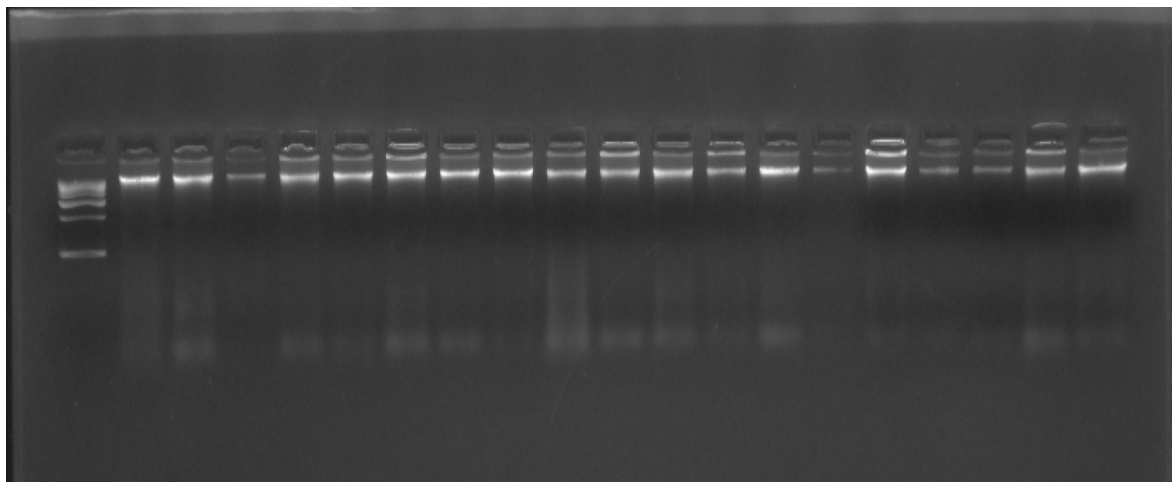


Plate 4: DNA isolates of PAEs degraders

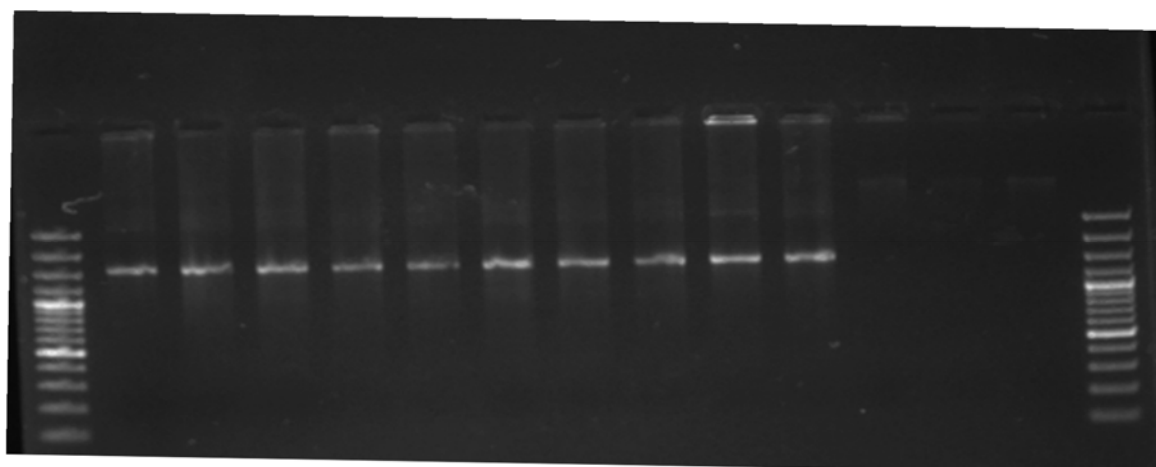


Plate 5: 16s rRNA of PAEs degraders



Plate 6: Laboratory scale set up for extraction of PAEs from soil

ppm after 30 days and again a drastic decline in DEHP was observed from 275 ppm at zero days to 92.8 ppm, 66.3 ppm and 51.8 ppm after 10, 20 and 30 days respectively.

The second bacterial culture was found to be most efficient in MS broth showed similar results in microcosm and only 49 ppm DEHP was present after 30 days of incubation as compared to *Pseudomonas sp. DEHPA2*. *Pseudomonas sp. DEHPB2* rapidly degraded DEHP from 276 ppm to 90.2 ppm, 64.1 ppm and 49 ppm in 10, 20 and 30 days respectively. The third bacterium *Pseudomonas sp. DEHPC2* also showed decline in DEHP from 275 ppm at initial to 50.2 ppm after 30 days of incubation. *Pseudomonas sp. DEHPC2* and *Pseudomonas sp. DEHPA2* were at par with each other for DEHP degradation.

The mixed inoculum (MI) was found to be most efficient in DEHP degradation where a decline in DEHP was observed from 276 ppm to 67.8 ppm, 54.4 ppm and 21 ppm at 10, 20 and 30 days of incubation.

Based on degradation pattern, the DEHP percentage degradation was also observed and (Table 4.17) shows a variation in percentage DEHP degradation among the bacterial isolates from 72.2 to 88.5% from 10 days to 30 days of incubation but a good DEHP percentage degradation was observed with mixed inoculum at 10, 20 and 30 days as compared to individual bacterial isolates.

The mixed inoculum showed 80.8% of DEHP degradation followed by *Pseudomonas sp. DEHPB2* 72.54%, *Pseudomonas sp. DEHPC2* 72.50% and *Pseudomonas sp. DEHPA2* 72.21% at 10 days. A slight variation was observed at 20 days where mixed inoculum showed 88.08% DEHP degradation followed by *Pseudomonas sp. DEHPB2* 81.08% but *Pseudomonas sp. DEHPA2* was found superior to *Pseudomonas sp. DEHPC2* showing 79.85% DEHP degradation as compared to 79.20% at 20 days. Again a different trend was observed where mixed inoculum showed 90.66% DEHP degradation followed by *Pseudomonas sp. DEHPC2* 88.5%, *Pseudomonas sp. DEHPB2* with 87.97% and *Pseudomonas sp. DEHPA2* showing 87.34% DEHP degradation at 30 days in soil microcosm. The results indicated the role of inoculated bacteria for the degradation of DEHP in soil microcosm (Figure 11).

4.8. Evaluation of selected DEP bacterial degraders in MS broth

Three bacterial isolates namely viz. *Achromobacter xylosoxidans* strain DEPA3, *Pseudomonas plecoglossicida* strain DEPB3 and *Enterobacter cloacae* strain

DEPC1 were selected for the degradation of DEP in MS broth with 300 mg L⁻¹ DEP as sole carbon source. Each bacterial culture was inoculated individually as well as mixed culture of all the three bacteria was also inoculated in MS broth and kept for incubation for 30 days. An un-inoculated broth was kept as control (Plate 7).

The results in (Tables 4.18, 4.19 & 4.20) show that the DEP degradation in flasks containing MS broth at 10, 20 and 30 days after inoculation. *Enterobacter cloacae* strain DEPC1 was found superior in degradation as compared to *Pseudomonas plecoglossicida* strain DEPB3 and *Achromobacter xylosoxidans* strain DEPA3 at 10 days of incubation. It was observed that from 282 ppm DEP on initial day, *Enterobacter cloacae* strain DEPC1 was able to degrade DEP resulting in 50.7 ppm as left over which was followed by strain *Pseudomonas plecoglossicida* strain DEPB3 55.3 ppm and strain *Achromobacter xylosoxidans* strain DEPA3 with 56.8 ppm DEP on 10th day from 281 ppm DEP at the start of experiment. After 20 days, although mixed inoculum showed maximum degradation with 24.2 ppm DEP presence in MS broth yet the individual culture was inoculated also showed variation in degradation in broth. *Achromobacter xylosoxidans* strain DEPA3 showed 33.2 ppm DEP, *Pseudomonas plecoglossicida* strain DEPB3 with 34.1 ppm and *Enterobacter cloacae* strain DEPC1 with 36.3 ppm DEP as 20th day of incubation. It was observed that strain *Achromobacter xylosoxidans* strain DEPA3 was superior over strains *Pseudomonas plecoglossicida* strain DEPB3 and *Enterobacter cloacae* strain DEPC1. Again after 30 days, degradation of DEP was rapid and with mixed inoculum 9.5 ppm DEP was observed which was the maximum and among the bacteria strain, *Enterobacter cloacae* strain DEPC1 was found superior over other two strains *Achromobacter xylosoxidans* strain DEPA3, *Pseudomonas plecoglossicida* strain DEPB3 for DEP degradation in MS broth. The reduction in pH of the broth was observed in all the inoculated flasks as compared to un-inoculated flasks and the range of pH reduction was observed as 6.1 -6.3.

Table 4.21 clearly indicated the role of bacterial culture as DEP percentage degradation in MS broth with time. It was observed that at 10th day mixed culture showed 82.77% degradation as compared to individual bacterial inoculated resulted in 74.67 to 75.34% degradation at 10th day. The percentage degradation increased with time where after 30 days mixed inoculum showed 96.14% degradation followed by

Table 4.17: Efficiency of bacteria for DEHP degradation in soil microcosm

S.N.	Isolates	% DEHP degradation in soil microcosm		
		10 d	20 d	30 d
1	<i>Pseudomonas sp. DEHPA2</i>	72.21	79.85	87.34
2	<i>Pseudomonas sp. DEHPB2</i>	72.54	81.88	87.97
3	<i>Pseudomonas sp. DEHPC2</i>	72.50	79.20	88.50
4	1+2+3	80.84	88.08	90.66

Table 4.18: Evaluation of selected DEP bacterial degraders in MS broth

S.No	Isolates	Days of incubation (ppm)			
		0 d	10 d	20 d	30 d
1.	<i>Achromobacterxylosoxidans</i> strain DEPA3	282	56.8	33.2	27.6
2.	<i>Pseudomonas</i> <i>plecoglossicida</i> strain DEPB3	282	55.3	34.1	31.7
3.	<i>Enterobacter cloacae</i> strain DEPC1	282	50.7	36.3	21.3
4.	1+2+3	281	34.1	24.2	9.5

Table 4.19: A X B X C Mean Table of 4.18

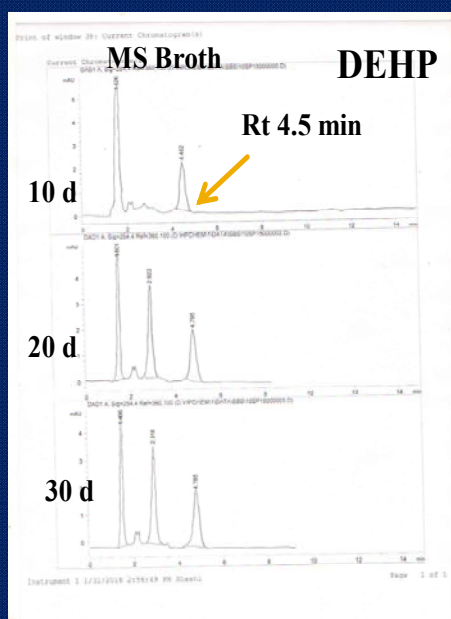
	A₁		A₂		A₃		A₄	
	B₁	B₂	B₁	B₂	B₁	B₂	B₁	B₂
C₁	281.733	281.733	282.233	282.233	282.000	282.000	281.033	281.033
C₂	280.933	56.833	281.467	55.333	281.567	50.733	280.933	35.133
C₃	280.000	33.233	280.533	34.100	280.833	36.267	280.433	24.233
C₄	280.367	27.633	280.067	21.667	279.833	22.000	279.567	9.500

Table4.20: SEM, SED and C.D. Table of 4.19

Factors	C.D.	SE(d)	SE(m)
Factor(A)	0.638	0.319	0.226
Factor(B)	0.451	0.226	0.160
Intraction A X B	0.902	0.451	0.319
Factor(C)	0.638	0.319	0.226
Intraction A X C	1.275	0.638	0.451
Intraction B X C	0.902	0.451	0.319
Intraction A X B X C	1.804	0.903	0.638

DEHP degradation chromatogram

In MS Broth



In Soil Microcosm

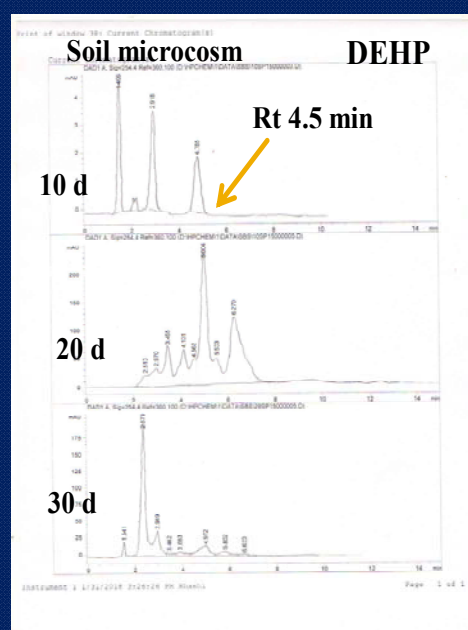


Figure 11: DEHP degradation chromatogram in MS Broth and soil microcosm



Plate 7: Degradation of DEP in MS broth using bacterial inoculation

Pseudomonas plecoglossicida strain DEPB3 (85.76%) while *Enterobacter cloacae* strain DEPC1 and *Achromobacter xylosoxidans* strain DEPA3 showed marginal differences with 84.70% and 84.91 % respectively (Figure 12).

4.9. Evaluation of DEP degrading bacteria in soil microcosm

The three selected bacteria namely viz. *Achromobacter xylosoxidans* strain DEPA3, *Pseudomonas plecoglossicida* strain DEPB3 and *Enterobacter cloacae* strain DEPC1 were further evaluated for the degradation of DEP in soil microcosm. The bacterial inoculation was done individually and also as mixed inoculum (MI) in 50 gm of soil having 300 ppm DEP at start of experiment. An un-inoculated control was also kept throughout the experiment (Plate 8).

The results in (Tables 4.22, 4.23 & 4.24) clearly indicate the role of inoculated bacteria in DEP degradation with incubation time. It was observed that the mixed culture consisting of equal ratio (1:1:1) of three bacterial cultures resulted in maximum degradation at 10, 20 and 30 days with 51.9 ppm, 32.3 ppm and 25.3 ppm DEP respectively from 271 ppm at the start of experiment. Similarly, among the individual culture, *Pseudomonas plecoglossicida* strain DEPB3 showed maximum degradation at 10 and 20 days with 72.4 ppm and 49.1 ppm DEP respectively from 271 ppm DEP at the start of experiment. This bacterial culture was followed by *Enterobacter cloacae* strain DEPC1 showing 75.2 ppm and 53.5 ppm at 10 and 20 days respectively while the third culture *Achromobacter xylosoxidans* strain DEPA3, was slightly slower in degradation showing 75.3 ppm and 54.6 ppm DEP at 10 and 20 days respectively from 271 ppm DEP at start of experiment.

Although throughout the experiment, mixed inoculum was found to be most efficient but the three cultures also showed good degradation resulting with variation in the presence of DEP after 30 days of incubation. *Enterobacter cloacae* strain DEPC1 showed maximum degradation with 31.3 ppm followed by *Pseudomonas plecoglossicida* strain DEPB3 with 32.6 ppm and followed by *Achromobacter xylosoxidans* strain DEPA3 with 34.3 ppm DEP from the range 271 ppm - 273 ppm DEP at the start of the experiment.

Further, the percentage degradation of DEP in soil microcosm was also estimated at different time intervals and it was observed that the bacterial culture *Achromobacter xylosoxidans* strain DEPA3 showed 66.2 to 81.6% DEP degradation

within 30 days while *Pseudomonas plecoglossicida* strain DEPB3 resulted in 67.31 to 82.24% DEP degradation and *Enterobacter cloacae* strain DEPC1 with 65.85 to 81.74% DEP degradation in soil microcosm from 10 days to 30 days of incubation (Table 4.25).

The mixed inoculum comprising of all these three bacterial culture (1:1:1) resulted as the most efficient degraders in 30 days where the range for percentage DEP degradation was observed as 75.43 - 92.39%. These results clearly indicate the role of mixed inoculum or a consortium for rapid degradation of DEP in soil microcosm.

Table 4.21: Efficiency of DEP degraders in MS broth

S.N.	Isolates	% DEP degradation in MS broth		
		10 d	20 d	30 d
1	<i>Achromobacter xylosoxidans</i> strain DEPA3	74.67	79.70	84.91
2	<i>Pseudomonas plecoglossicida</i> strain DEPB3	75.34	80.80	85.76
3	<i>Enterobacter cloacae</i> strain DEPC1	74.96	80.45	84.70
4	1+2+3	82.77	88.63	96.14

Table 4.22: Evaluation of DEP degrading bacteria in soil microcosm

S.No	Isolates	Days of incubation (ppm)			
		0 d	10 d	20 d	30 d
1.	<i>Achromobacter xylosoxidans</i> strain DEPA3	271	75.3	54.6	34.3
2.	<i>Pseudomonas plecoglossicida</i> strain DEPB3	271	72.4	49.1	32.6
3.	<i>Enterobacter cloacae</i> strain DEPC1	273	75.2	53.5	31.3
4.	1+2+3	271	51.9	32.3	25.3

Table 4.23: A X B X C Mean Table of 4.22

	A₁		A₂		A₃		A₄	
	B₁	B₂	B₁	B₂	B₁	B₂	B₁	B₂
C₁	271.200	271.200	271.367	271.367	272.600	272.600	271.233	271.233
C₂	270.700	75.267	271.000	72.400	272.133	75.167	270.433	51.933
C₃	270.667	54.600	271.167	49.133	272.133	53.500	270.367	32.267
C₄	269.567	34.267	270.000	32.600	271.400	31.333	270.100	25.333

A₁= Isolates DEPA3, A₂= Isolates DEPB3, A₃= Isolates DEPC1, A₄=

Isolates A1+B1+C1, B₁= Control, B₂=Inoculate, C₁= 0 day, C₂= 10 days, C₃= 20 days, C₄= 30 days

Table 4.24: SEM, SED and C.D. Table of 4.23

Factors	C.D.	SE(d)	SE(m)
Factor(A)	0.768	0.384	0.272
Factor(B)	0.543	0.272	0.192
Intraction A X B	1.086	0.544	0.384
Factor(C)	0.768	0.384	0.272
Intraction A X C	1.536	0.769	0.544
Intraction B X C	1.086	0.544	0.384
Intraction A X B X C	2.173	1.087	0.769

Table 4.25: Efficiency of selected bacteria for DEP degradation in soil microcosm

S.N.	Isolates	% DEP degradation in soil microcosm		
		10 d	20 d	30 d
1	<i>Achromobacterxylosoxidans</i> strain DEPA3	66.25	75.89	81.16
2	<i>Pseudomonas plecoglossicida</i> strain DEPB3	67.31	76.77	82.24
3	<i>Enterobacter cloacae</i> strain DEPC1	65.85	75.41	81.74
4	1+2+3	75.43	80.28	92.39

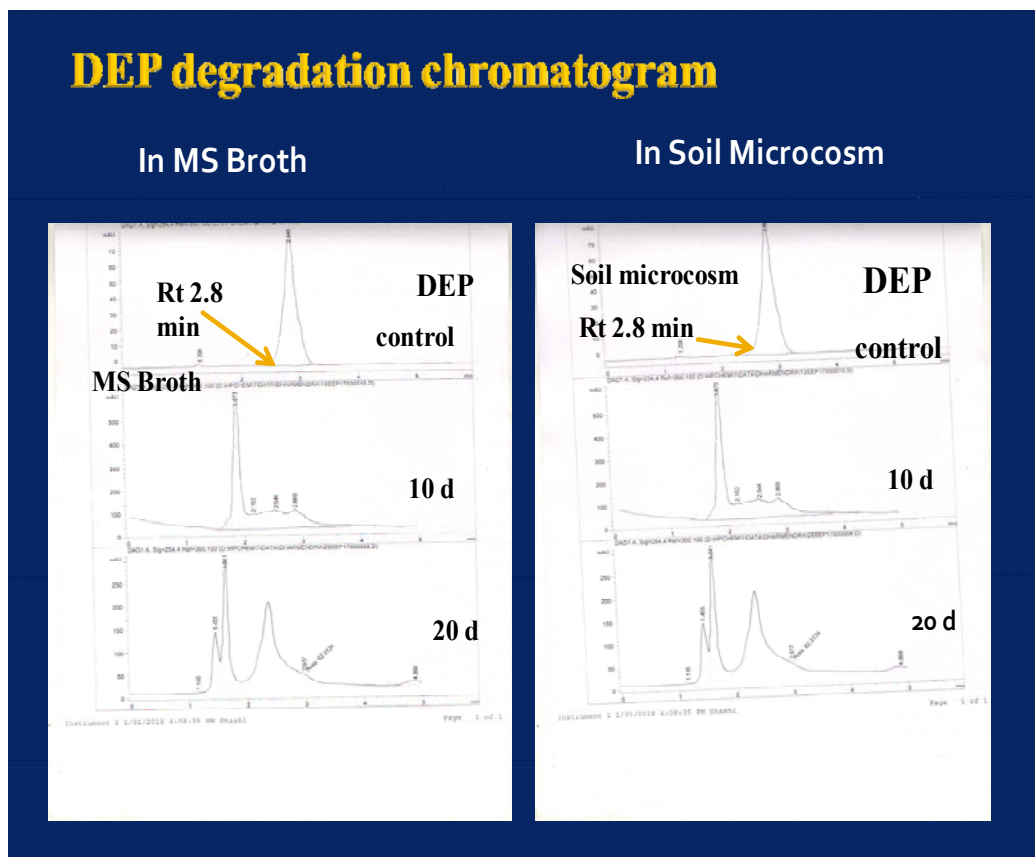


Figure 12: DEP degradation chromatogram in MS Broth and soil microcosm



Plate 8: Soil microcosm set up for DEP degradation

5. DISCUSSION

5.1. Physico-chemical properties of soil

Phthalate esters (PAEs) have wide application as plasticizers of polyvinyl chloride. They are also used for manufacturing plastic toys, bags, pipes, day to day household products like flooring, lubricants (Crinnian *et al*; 2010) and their application has increased from last 20 years. It has been observed that phthalate esters production is increasing worldwide at constant level and it has been estimated approximately 4.3 million tonnes PAEs per year are being produced (Peijneburg and Struijs, 2006). Since they are added in plastics they are termed as ubiquitous environmental pollutants (Tan *et al*; 2016 and Zeng *et al*; 2008) and pollute soil, air, and water (Cheng and Chang, 2016). In agriculture, there is wide use of plastic film which is difficult to degrade (Lu *et al*; 2009) and it has been observed that PAEs from soil enter food chain (Cai *et al*; 2008). Wang *et al*. (2016) have reported that due to increase of plastics film in soil, there is simultaneous decrease in biomass carbon and nitrogen, microbial diversity is also negatively affected. It has been further studied that PAEs can also damage human health leading to disturbance in endocrine system and carcinogenic in animals (Howdeshell *et al*; 2007). Many researchers have also reported that DEHP can cause reproductive toxicity, embryo developmental toxicity and could be immunotoxic (Heudorf *et al*; 2007).

To decontaminate the phthalate esters from soil, bioremediation is one of the alternatives where efficient microorganisms could be applied in soil especially for rapid degradation of phthalate esters.

In the present study, an attempt has been made to collect soil samples contaminated with phthalate esters at centre of CPCT, ICAR- Indian Agricultural Research Institute, New Delhi. A number of studies on natural water, waste water, and soil have been carried out till date and many bacteria have been isolated from rivers, soils but mainly for degrading PAEs with shorter alkyl chains like DBP while PAEs with longer alkyl chains like DEHP are poorly degraded under aerobic and anaerobic conditions (Chen *et al*; 2007). Previous studies suggest that the soil or sludge samples used for isolation were collected from heavily contaminated with plastic pollutants mainly rivers and factories (Feng *et al*; 2002, Ahn *et al*; 2004; and Chen *et al*; 2007). Hence, soil samples collected for our present study were also from

plastic contaminated sites at centre of CPCT, New Delhi. A total of three samples from three different sites were collected and labelled as DK 1 (Inside green house), DK 2 (Mulching site) and DK 3 (Outside greenhouse) respectively.

Muhr *et al.* (1965) have suggested the limits for dissolvable salts in soil where $EC < 1.0 \text{ dSm}^{-1}$ is characterized as normal and it was also observed that conductivity of soil vary from $0.20\text{-}0.52 \text{ dSm}^{-1}$. Mohanty *et al.* (2000) had reported that IARI soils are non saline with $EC \text{ } 0.08\text{-}1.04 \text{ dsm}^{-1}$ and $pH \text{ } 5.89\text{-} 9.10$. Similar results were also obtained with four soil samples where EC was in range $0.45\text{-} 0.49 \text{ dSm}^{-1}$ and maximum EC was obtained with soil sample DK 3 followed by DK 2 and DK 1.

All the collected soil samples were analysed for physico-chemical parameters. The soil was found sandy loam in texture and samples showed marginal differences in pH , Electrical conductivity (EC), organic carbon (OC), P_2O_5 , K_2O , sulphur, boron, iron, manganese, and zinc content.

The pH range of three collected samples was observed as $7.1\text{-}7.65$ indicating neutral to slightly alkaline nature. These results were in accordance with the classification given by Brady (1985) where $pH \text{ } 6.6 \text{ to } 7.3$ is for neutral, $pH \text{ } 7.4 \text{ to } 7.8$ is mildly alkaline and $7.9\text{-}8.4$ is moderately alkaline.

Soil organic carbon is another important soil fertility factor and the build-up of organic carbon is time-consuming and it takes years to increase organic carbon in soil. The experiments carried by Ladha *et al.* (2003) reported that while carrying out long-term fertility experiment in rice-wheat cropping system in north-west India for 25- 30 years, the organic carbon remained static and Bhattacharya *et al.* (2007, 2008) also reported that there was an increase in SOC in 25 years from 1980-2005 especially in Indo-Gangetic plains and black soil. The results obtained with contaminated soil samples DK 1, DK 2 and DK 3 shows organic carbon $0.1\text{-} 0.6\%$ indicating poor organic carbon content.

Muhr *et al.* (1965) reported the extractable potassium content in various soils and observed that extractable potassium as an average is 201.7 Kg ha^{-1} in soils and the range is $134.6\text{-} 310.4 \text{ Kg ha}^{-1}$. It was also reported that 96% of soil samples are medium in extractable potassium having $125\text{-}300 \text{ Kg ha}^{-1}$. Similarly, the three soil samples were found to be containing higher potassium content than an average and

medium soil. The extractable potassium was observed within 486.5 kg ha⁻¹ to the maximum 626.2 kg ha⁻¹ where DK 3 (mulching site) showed highest K₂O content followed by DK 2 (560.4 kg ha⁻¹) and lowest in DK 1 (486.5 kg ha⁻¹).

The available phosphorus content varied from 8.2 to 25.0 kg ha⁻¹ with a mean value of 12.8 kg ha⁻¹ and the basis of the limits suggested by Muhr *et al.* (1965), most of the soil samples (94 %) were low (< 20 P₂O₅ kg ha⁻¹) in available phosphorus status and rest were under medium (20-50 kg ha⁻¹) category.

The presence of Zn was found maximum in sample DK 2 as compared to DK 1 and DK 3 and the zinc range was observed as 0.5 to 18.8 kg ha⁻¹ while sulphur, boron, iron, and manganese were present as 4.84 to 5.41 µg kg⁻¹, 0.416 to 2.12 µg kg⁻¹, 0.63-16.74 µg kg⁻¹ and 3.30 to 5.41 µg kg⁻¹ soil respectively. Hariram and Dwivedi (1994) have classified soil in terms of available sulphur as deficient (< 10 ppm), medium (10-20 ppm) and sufficient (>20 ppm). Results obtained clearly indicate that all the three samples sufficient in sulphur having >20 ppm.

Boron content was also found maximum in sample DK 2 (2.125 µg kg⁻¹) followed by DK 3 and DK 2. Iron content was observed highest in DK 3 (16.6 µg kg⁻¹) and the range observed was 0.06 to 16.6 µg kg⁻¹ while manganese content was maximum in soil sample labelled as DK 2 (54.1 µg kg⁻¹) followed by DK 1 (33.3 µg kg⁻¹) and DK 3 (26.3 µg kg⁻¹).

All the observation showed variation in different nutrients content in three soil samples DK1, DK 2 and DK 3 but most of nutrients were found in high concentration in sample DK 2 and based on the previous studies the results collaborate with earlier work with slight variation due to the source and degree of phthalate ester contamination.

5.2. Fortification of soil to recover DEP and DEHP

According to USEPA, 6 mg L⁻¹ DHEP is permissible in water and this was the maximum concentration admissible and the threshold limit value of DEHP, DEP and DMP are 0.55, 0.45 and 5.0 mg L⁻¹ respectively. Hence, keeping these standards in mind in the present study optimization to form a standard protocol for the estimation of residual DEP and DEHP from soil and MS broth, an experiment was carried out where both soil and MS broth fortified with three different concentrations

mainly 10 ppm, 100 ppm and 300 ppm DEP and DEHP respectively. Results obtained were found within the permissible limits that when DEP concentration was increased in broth from 10 ppm to 300 ppm the recovery of same compound was found in range 94.8 to 95.3% respectively. Similarly with an increase of DEP concentration from 100 ppm to 300 ppm showed no effect on recovery resulting 95.3% recovery at 100 ppm as well as 300 ppm while DEHP was also studied for recovery in MS broth and soil and it increased from 90.93 to 91.33% along with increase in DEHP concentration from 10 ppm to 300 ppm in MS broth and it also increased in soil showing 93.4 to 93.76% recovery.

5.3. Morphological characterization of DEP and DEHP degrading bacterial isolates

Isolation of phthalate degrading microorganisms using enrichment techniques: The main objective was to isolate phthalate degrading bacteria mainly for DEP and DEHP which are PAEs with longer alkyl chains and are poorly degraded under aerobic and anaerobic conditions (Chen *et al*; 2007). Here, DEP and DEHP were used as sole source of carbon and on visual observation, ninety bacteria were isolated successfully. Karegoudar *et al.* 1984 have also reported microorganisms belonging to group *Pseudomonas*, *Micrococcus*, *Aspergillus* and *Bacillus* sp. capable of degrading by-products of phthalate esters. All the three soil samples after enrichment were subjected to serial dilution, spread plate, and streak plate to obtain pure culture so as to further investigate for morphological, biochemical and molecular characterization.

A gram-positive *Microbacterium* sp. strain CQO110Y showing round morphology, smooth and glossy surface and yellowish colouring were isolated by Chen *et al*; (2007). Similarly, all the ninety isolates were purified to single colony for morphological characterization and based on morphological characterization, 18 isolates were selected for further studies. All the isolates were found to be rod-shaped except DEP B2 which was cocci. Most of the colonies were whitish in colour, however, variation in white colour was observed from dull, creamy white to off-white while two colonies were shiny with blackish shade. Margins of colonies varied from circular, irregular to punctiform with entire, curled and undulated margin. Another gram-positive, motile, rod *Bacillus* strain T-36 was isolated from sea mud in the Taganoura harbor, Japan (Sakagami *et al*; 1982).

Some of colonies were pigmented and the margins of colonies were either flat or raised. These bacteria were designated as DEP A1 to DEP A15, DEP B1 to DEP B15 B1, DEP C1 to DEP C15 for DEP degradation DEHP A1 to DEHP A15, DEHP B1 to DEHP B15 and DEHP C1 to DEHP C15 for DEHP degradation.

Hence, a total of 18 bacterial isolates based on morphology were finally selected for further studies and similar study was also carried by Kurane *et al.* 1980 where they have reported that the bacterial isolates capable of utilizing phthalate esters were mostly Gram-positive like *Rhodococcus erythrophiles* and *Corynebacterium sp.* while *Pseudomonas* was rarely seen.

The results obtained are in contrast with earlier observation as most of the isolates belong to the genus *Pseudomonas* and this difference may be due to the source of isolation like soil, sewage from plastic factory. Similar to present studies Sakagami *et al.* (1982) have also reported Gram-negative rods belonging to the genus *Pseudomonas* and *Alcaligenes*. All the nine isolates were found to be Gram-negative rods for DHEP degradation whereas eight isolates as DEP degraders were gram-negative rods and DEP A2 were observed as cocci.

5.4. Biochemical characterization of DEP and DEHP degrading bacterial isolates

The nine DEP and DEHP degrading isolates were studied for biochemical characterization mainly hydrolysis of urea, starch, esters, and production of cytochrome oxidase and catalase. Results obtained were similar to the investigation carried by Pradeep *et al.* (2015) where they found variation among the various microbial isolates from contaminated soil of Kerala. Similarly all the eighteen bacteria were able to produce cytochrome oxidase, catalase, amylase, and esterase but seven DEP degraders namely DEP A1, DEP A2, DEP A3, DEP B2, DEP B2, DEP B3, and DEP C1 and five DHEP degraders DEHP A2, DEHP B1, DEHP B2, DEHP B3, and DEHP C2 were unable to produce the enzyme urease for hydrolysis of urea. All these enzymes are needed for catalyzing the hydrolysis of ester bonds in PAEs for the formation of monoesters (Liang *et al.*; 2008)

5.5. Growth of bacterial isolates at different concentrations of DEP and DEHP:

Xia *et al.* (2006) reported that when the initial concentration of PAEs is less than 50 ppm or more than 200 ppm the growth of microbes is limited and the biodegradation rate also decreases. PAEs provide the sole carbon source to microbes

and if the initial PAEs concentration is insufficient, the microbes cannot get enough food supply so their growth will be restrained. On the other hand, PAEs are toxic substances; when the initial concentration is more than 200 ppm, they will damage the microbes, and limits their growth. The biodegradation rate of PAEs also decreases when the concentration of PAEs is too high.

Similar results were obtained in the present study when the concentration of both compounds DEP and DEHP were varied from 100-700 ppm and all the bacterial isolates nine each for DEP and DEHP were grown on different concentration from 100 to 700 ppm of respective compound in mineral salt medium broth for the estimation of total protein in μg per mL. Results obtained revealed that all the nine isolates were able to grow in MS broth with variation at different concentration of DEHP and DEP. On the whole, the protein concentration varied from 48.00 to 68.3 ppm and the bacterial isolates DEHP A2, A3 and B1 showed protein concentration in range 67.00 to 4.00 ppm with simultaneous increase in DEHP from 100 to 700 ppm. On the basis of protein concentration, DEHP A1, DEHPB2, and DEHP C2 were finally selected for studying DEHP degradation and DEP A2, DEP B2 and DEP C3 as DEP degrader for final molecular characterization.

5.6. Molecular characterization of bacterial isolates using 16s rRNA and phylogenetic analysis:

It has been observed that DEHP contamination in the environment is always complex and there exist several kinds of PAEs and polychlorinated biphenyls (PCBs) simultaneously (Fiandanese *et al*; 2016) reports have been studied for he biodegradation process of PAEs (including DEHP) where it is stated that PA is the central intermediate and transforms first into another key intermediate (Ren *et al*; 2018) which is a recalcitrant compound. This compound is being suspected to cause cancer and kidney damage (Zhou *et al*; 2016).

Further, it has been established that all bacteria cannot, transform DEHP into certain intermediates like PA or BA, For example, *Gordonia sp.* Dop5 failed to use PA(Sarkar *et al*; 2013). Conversely *Rhodococcus sp.* HS-D2 (Zhang *et al*; 2016), *Bacillus subtilis* No.66(Quan *et al*; 2005), *Gordonia alkanivorans* YC-RL2 (Nahurira *et al*; 2017), *Providencia sp.* 2D (Zhou *et al*; 2016) and *Arthrobacter C21* (Wen *et al*; 2014) completely degraded PAEs. As compared with the reported

strains, *Rhodococcus ruber* YC-YT1 is capable of efficiently metabolizing all the intermediates of phthalate esters like DEP, DEHP, and DOP.

5.7. Microbial degradation of DEHP in MS broth

Wen *et al.* (2014) reported phthalate ester degrading *Arthrobacter* sp. isolated from constructed wetland soil could grow with DBP, DMP, DEP, and DEHP as sole carbon source. Another report by Wang *et al.*; 2012 showed that *Arthrobacter* sp. 2H₂ which was isolated from mangrove sediments utilized phthalates with shorter alkyl chains like DMP and DBP but unable to grow on DOP.

A similar results were also reported by Wu *et al.* 2010 where *Arthrobacter* sp. JDC-32 could not degrade DOP. Further, it was reported that co-culture of non DOP degrading *Arthrobacter* sp and DOP degrading *Gordania* sp could degrade DOP completely (Wu *et al.*; 2010, 2011).

In the present study, similar results were obtained where the three isolates viz. *Pseudomonas* sp. DEHPA2, *Pseudomonas* sp. DEHPB2, *Pseudomonas* sp. DEHPC2 were able to grow in MS broth containing 300mg L⁻¹ DEHP. The degradation was confirmed by lowering the pH from 6.1 to 6.3 of medium and estimating the left out DEHP after 30 days incubation after inoculation. The maximum degradation was observed with mixed culture where percentage degradation varied significantly showing 79

.8 - 96.6% degradation after 30 days incubation.

5.8. Evaluation of selected bacteria for DEHP degradation in soil microcosm

Microbial activity is the major mechanism responsible for the degradation of phthalate esters in soils and sediments and is more effective under aerobic than anaerobic conditions (Yuan *et al.*; 2002).

Chang *et al.* (2004) have assessed the environmental fate of eight different phthalate esters and reported that their degradation tended to be more difficult with increasing alkyl-side chain length. Thus, the average half-life for biodegradation was 14.8 days for DEHP and 2.9 days for di-butyl-phthalate (DBP) using river sediments as the inoculum, under aerobic conditions.

Madsen *et al.* (1999) also reported the ability of indigenous microorganisms to degrade DEHP in sludge-amended soil was very poor and it was estimated that more than 41% of the DEHP added to the soil was still present after 1 year.

Bioaugmentation is known as the specific enhancement of biological activity through the addition of microorganisms with the desired catalytic capabilities and it has been studied extensively for the remediation of environmental pollution by recalcitrant chemicals (Salanitro *et al.*; 2000 and Bento *et al.*; 2005).

Research has shown that bioaugmentation through the introduction of bacteria can change the structure of the microbial community, which in turn may affect biogeochemical cycles. Therefore, before selecting bioaugmentation for *in situ* remediation, the effect of the introduced degradative bacteria on the surrounding environment must be clarified.

The decrease in DEHP degradation in the presence of soil may be due to poor bioavailability (Roslev *et al.*, 1998; Cartwright *et al.*, 2000). Recently, Quan *et al.* (2005) showed that by adding 8% of culture medium to soil, *Bacillus subtilis* No. 66 could degrade 80% of the added DEHP in 5 days, while little or no degradation was observed in the absence of culture medium. After incubation for 35 days, >50% of the initial concentration of DEHP (200 mg kg⁻¹) was degraded in all examined soils. Similar levels of degradation have been reported in other studies (Cartwright *et al.*, 2000 and Zhu *et al.*, 2018).

A number of bacterial strains that can efficiently degrade DEHP alone or by cooperation have been isolated from environmental sites (Chen *et al.*, 2007, Kurane *et al.*, 1984 and Nishioka *et al.*; 2006).

These results suggest that bacteria have adapted to DEHP contamination and have acquired the ability to detoxify or degrade this compound. Although a few fungal species have also been shown to possess DEHP degradation capability (Aguilar-Alvarado *et al.*; 2015 and Kim *et al.*; 2003), the current study did not find a correlation between fungal population and DEHP degradation, possibly reflecting a less important role for fungi in DEHP degradation as well as the source of isolation.

Similarly the three bacterial isolates *Pseudomonas* sp. DEHPA2, *Pseudomonas* sp. DEHPB2 and *Pseudomonas* sp. DEHPC2 have inoculated in soil for degradation of DEHP individually as well as a consortium in 50 gm soil containing 300 ppm DEHP. The results obtained were in accordance with the earlier work stating varied degree of degradation of phthalate esters. The mixed inoculum (MI) was found to be most efficient in DEHP degradation where the decline in DEHP was observed from 276 ppm to 67.8 ppm, 54.4 ppm and 21 ppm at 10, 20

and 30 days of incubation and 72.2 - 88.5% degrading efficiency was observed clearly highlighting the role of inoculated bacteria as compared to uninoculated medium.

5.9. Evaluation of selected DEP bacterial degraders in MS broth

Phthalate isomers and their esters are present in the environment. Due to persistence in nature, microbes have evolved and adapted new pathways to degrade these compounds. Bacterial degradation is efficient as these compounds are either biotransformed or mineralized completely. Phthalate esters are first hydrolysed to their respective phthalate isomers by esterases. In *Gordonia* sp. strain MTCC 4818, esterase was shown to be inducible and had broad substrate specificity (Chatterjee *et al*; 2003)

Some microbes can directly hydrolyzed esterified phthalate and terephthalate to respective phthalate isomers by *trans*- esterification (Cartwright *et al*; 2000 and Li *et al*; 2000). *Sphingomonas* sp. DEP-AD1 could grow by hydrolyzing diethyl phthalate, dibutyl phthalate, and diethyl hexyl phthalate; but could not metabolize aromatic ring of phthalate and benzoate suggesting that the organism utilizes aliphatic metabolites generated after de-esterification (Fang *et al*; 2007)

The initial step in the metabolism of phthalate esters is hydrolysis of ester bond by esterase/hydrolase to yield monoesters and then phthalate isomer (Keyser *et al*; 1976, Maruyama *et al*; 2005, Niazi *et al*; 2001 and Quan *et al*; 2005). Esterases/hydrolase are inducible and show broad substrate specificity (Wang *et al*; 2003) while few are specific (Wang *et al*; 2006)

The ability of organisms to degrade one or two specific phthalate isomer(s) suggest the probable lack of genetic material that code for degradative enzymes of another isomer (s). Hence, bacterial cocktail consisting of organisms specific carbon and efficient for various isomers are preferred for effective bioremediation. For example, mixed culture of *Pseudomonas* and *Bacillus* metabolizes terephthalate at high concentrations (8 g/l) from waste waters completely as compared to partial utilization by a single strain (Kimura *et al*; 2001)

Herada and Koiwa, (1977) enriched *Alcaligenes*, *Arthrobacter* and *Corynebacterium* strains using three isomers of phthalate as carbon source. As a single isolate, none of them could grow on mixture of three isomers, however, as mixed culture they could degrade all isomers efficiently.

The bacterial consortia mineralizes low molecular weight phthalate esters faster than higher molecular weight counterparts (Sugatt RH *et al*; 1984). Consortia of *Sphingomonas* sp. DK4 and *Corynebacterium* sp. O18 have ability to degrade 8 different phthalate esters, however, degradation efficiency enhanced when both strains were present simultaneously (Cartwright *et al*; 2000).

Pseudomonas fluorescens, *Pseudomonas aureofaciens* and *Sphingomonas paucimobilis* as consortia can hydrolyze dimethyl phthalate to phthalate via monomethyl phthalate, which is further metabolized as the carbon source whereas single isolate failed to utilize it as the carbon source (Wang *et al*; 2003)

Three bacterial isolates namely viz. *Achromobacter xylosoxidans* strain DEPA3, *Pseudomonas plecoglossicida* strain DEPB3 and *Enterobacter cloacae* strain DEPC1 were selected for the degradation of DEP in MS broth with 300 mg L⁻¹ DEP as sole carbon source.

Similar to earlier work *Enterobacter cloacae* strain DEPC1 was found superior in degradation as compared to *Pseudomonas plecoglossicida* strain DEPB3 and *Achromobacter xylosoxidans* strain DEPA3 at 10 days of incubation. After 20 days, although mixed inoculum showed maximum degradation with 24.2 ppm DEP presence in MS broth yet the individual culture was inoculated also showed variation in degradation in broth. *Achromobacter xylosoxidans* strain DEPA3 showed 34.2 ppm DEP, *Pseudomonas plecoglossicida* strain DEPB3 with 341 ppm and *Enterobacter cloacae* strain DEPC1 with 36.3 ppm DEP as 20th day of incubation. Again after 30 days, degradation of DEP was rapid and with mixed inoculum 9.5 ppm DEP was observed which was the maximum and among the bacteria strain *Enterobacter cloacae* strain DEPC1 was found superior over other two strains *Achromobacter xylosoxidans* strain DEPA3, *Pseudomonas plecoglossicida* strain DEPB3 for DEP degradation in MS broth.

The reduction in pH of the broth was observed in all the inoculated flasks as compared to un-inoculated flasks and the range of pH reduction was observed as 6.1-6.3. Result also indicated the role of bacterial culture as DEP percentage degradation in MS broth with time. *Pseudomonas plecoglossicida* strain DEPB3 (85.76%) while *Enterobacter cloacae* strain DEPC1 and *Achromobacter xylosoxidans* strain DEPA3 showed marginal differences with 84.70 and 84.91 % respectively.

5.10. Evaluation of DEP degrading bacteria in soil microcosm

Many bacterial species have been isolated from activated sludge, mangrove sediment, soil and solids, wastewater, river sludge etc. with the ability to degrade PAEs among different bacterial strains included are from about 25 genera such as *Pseudomonas*, *Sphingomonas*, *Achromobacter*, *Rhodococcus*, *Gordonia*, *Cornebacterium* and *Agrobacterium* (Wu *et al*; 2011, Wang *et al*; 1995, 2000, Jianlong *et al*; 1995; Shelton *et al*; 1984, Roslevet *et al*; 2007, Xu *et al*; 2007, Liang *et al*; 2008 and Lu *et al*; 2009).

Cartwright *et al*; (2000) reported that DEP added to soil at concentrations of 10 mg g⁻¹ or below was up to 90% biodegraded in 59 days, and Jianlong and Xuan (2004) showed DEP degradation in sludge-amended soils with a half-life of 3.7 days. In natural environments, complete degradation of complex organics, like phthalates, is always carried out syntrophically by several members of microorganisms (Gu *et al*; 2005).

The biodegradation of phthalates primarily involves the sequential hydrolysis of ester linkage, which results in monoesters and, subsequently, PA, while forming alcohols simultaneously. Thus, the microbial assimilation of phthalates requires diverse metabolic genes and enzymes, indicating a single organism is unlikely able to completely mineralize phthalates (Staples *et al*; 1997). As examples, in the degradation of DMP isomers by *Rhodococcus ruber*, their corresponding intermediates, mon-methyl phthalate (MMP) isomers, were not further degraded (Li *et al*; 2005); BBP metabolites, monobutyl phthalate (MBP) and mono-benzyl phthalate, could not be further degraded by the BBP-degrading bacteria *Gordonia sp.* MTCC 4818 (Chatterjee and Dutta 2003) while Engelhardt and Wallnofer (1978) also reported that a DBP degrading bacterium could not degrade MBP.

So far, mixed culture consortia have been shown to completely mineralize phthalates. Wang *et al.* (2004) reported that DMP was completely mineralized by two consortia of microorganisms; one comprised of *Pseudomonas fluorescens*, *P. aureofaciens* and *Sphingomonas paucimobilis*, whereas the other comprised of *Xanthomonas maltophilia* and *S. paucimobilis*. DMP was also found to be serially degraded by a coculture of an *Arthrobacter sp.* and *S. paucimobilis*. The former species was responsible for transforming DMP into MMP, which was utilized by the latter species.

BBP was completely mineralized by a mixed culture consortium, in which *Arthrobacter* sp. WY was able to metabolize BBP into MbuP, MBzP, and PA, while *Acineobacter* sp. FW was capable of degrading de-esterified alcohol and thus completed BBP mineralization (Chatterjee and Dutta 2008).

Homologous bacterium *Acinetobacter* sp. strain M673 owns the ability to hydrolyze many kinds of PAEs with different chains, but it could not completely utilized them (Wu *et al.*, 2013).

Some other strains, *Sphingomonas* sp. DK4, *Corynebacterium nitrilophilus* G11, and *Acinetobacter wolffii* could also use DBP or DEHP as the only carbon source, but degrading efficiency was not as high as strain LMB-5 (Hashizume *et al.*, 2002; Zeng *et al.*, 2004 and Chao *et al.*, 2006). *Pseudomonas fluorescens* B-1 had the ability to utilize DBP, and it can completely remove 10 mg L⁻¹ DBP with a 96 hr incubation (Xu *et al.*, 2005). Degradation of DBP (100 mg L⁻¹) by *Rhodococcus ruber* reached to 55% after 10 d of incubation (Li *et al.*, 2006) and compared with these strains, strain LMB-5 was more efficient in degrading phthalate.

Gordonia sp. strain P8219 could utilize DEHP as a sole source of carbon, and DEHP was quickly degraded with the growth of cells and disappeared in 60 h (Nishioka *et al.*, 2006). Its ability to degrade DEHP was much better than that of the strain LMB-5. Prasad and Suresh (2015) have studied a strain *Variovorax* sp. BS1 which could biodegrade 600 mg L⁻¹ DMP; DMP was totally consumed after 30 hr of incubation.

A novel study of *Rhizobium* sp. LMB-1 has shown its biodegrading ability of DBP, DMP, DEP, and DEHP, and the conjectural degradation pathway of DBP was similar to the metabolic pathway of strain LMB-5 (Tang *et al.*, 2016).

In present investigation also the three selected bacteria namely viz. *Achromobacter xylosoxidans* strain DEPA3, *Pseudomonas plecoglossicida* strain DEPB3 and *Enterobacter cloacae* strain DEPC1 were found to be degrading DEP in soil, similar to earlier work DEP degradation is due to inoculated bacterial culture with varied rate.

Pseudomonas plecoglossicida strain DEPB3 showed maximum degradation at 10 and 20 days with 72.4 ppm and 49.1 ppm DEP respectively from 271 ppm DEP at the start of experiment while *Enterobacter cloacae* strain DEPC1 showing

75.2 ppm and 53.5 ppm at 10 and 20 days respectively and *Achromobacter xylosoxidans* strain DEPA3, was slightly slower in degradation showing 75.3 ppm and 54.6 ppm DEP at 10 and 20 days respectively from 271 ppm DEP at start of experiment.

Enterobacter cloacae strain DEPC1 showed maximum degradation (31.3 ppm) as compared to *Pseudomonas plecoglossicida* strain DEPB3 (32.6 ppm) and *Achromobacter xylosoxidans* strain DEPA3 (34.3 ppm) DEP from 271 -273 ppm DEP at the start of the experiment.

Achromobacter xylosoxidans strain DEPA3 showed 66.2 to 81.6% *Pseudomonas plecoglossicida* strain DEPB3 resulted in 67.31 to 82.24% and *Enterobacter cloacae* strain DEPC1 with 65.85 to 81.74% DEP degradation in soil microcosm from 10 days to 30 days of incubation.

The mixed inoculum comprising of all these three bacterial cultures (1:1:1) resulted as the most efficient degraders in 30 days where the range for percentage DEP degradation was observed as 75.43- 92.39%. These results clearly indicate the role of mixed inoculum or a consortium for rapid degradation of DEP in soil microcosm and also collaborates with work done by earlier researchers with isolates from contaminated river, sludge, etc.

6. SUMMARY AND CONCLUSION

Phthalate esters are compounds mainly consisting of Di alkyl or alkyl aryl esters of 1, 2- benzene, Di carboxylic acid (Phthalic acid). Various PAEs like bis (2-ethylhexyl) phthalate (DEHP), Di butyl phthalate (DBP), Di ethylphthalate (DEP) and Di methyl phthalate (DMP) are being used worldwide for various commercial products.

These PAEs have been used commonly as plasticizers in plastic products like polyvinyl chloride (PVC) resins, adhesives, and cellulose film coatings, flooring, wall covering, medical devices and furniture. PAEs are also extensively used in plastic film of greenhouse and pipes used for irrigation. Plastic film is mainly used for the walls of polytunnels or for mulching at the soil surface in intensive protected vegetable cultivation and fertilizers etc. PAEs can also lead to atmospheric or water pollution by evaporation, run off, leaching, absorption, deposition, and drainage in soil. It is known that when they are used as plasticizers, PAEs are not chemically bonded to the plastics polymer and therefore eventually can migrate from the plastics into the environment.

PAEs and their metabolites which are produced during its degradation have been reported to cause reproductive and developmental disorder. PAEs are causing estrogenic, hepatotoxic, teratogenic, carcinogenic and endocrine disruptive effects *in vivo*. They are generally found in soil, water, air, food products, animal and human body as they can easily enter food chain.

Bioremediation involves the degradation of pollutant with the help of the enzymes present in aerobic, anaerobic or facultative microorganisms. Many bacterial species have been isolated from activated sludge, mangrove sediment, soil and solids, wastewater, river sludge, etc. with the variation in their ability to degrade PAEs.

Among different bacterial strains, about 25 genera such as *Pseudomonas*, *Sphingomonas*, *Rhodococcus*, *Enterococcus*, *Gordonia*, *Cornebacterium*, and *Agrobacterium* are found most prominent in degradation.

- The soil samples were collected from three different contaminated sites at Centre for Cultivated Protection Technology (CPCT) IARI, New Delhi, India as it was found to be contaminated with phthalate esters. All the collected

samples were analyzed for physico-chemical parameters such as pH, Electrical conductivity (EC), organic carbon (OC), P_2O_5 , K_2O , sulphur, boron, iron, manganese, and zinc content.

- The pH range of three collected samples was observed as 7.1-7.65 showing neutral to slightly alkaline nature. EC of the soil samples was observed in the range 0.457-0.496 dS m^{-1} .
- The organic carbon percentage range was observed from 0.1-0.6%. The extractable potassium was observed within 486.5 kg ha^{-1} to the maximum 626.2 kg ha^{-1} while phosphorus content (P_2O_5) was obtained in the range of 15.53 to 19.2 kg ha^{-1} . The zinc range was observed as 0.5-18.8 kg ha^{-1} while sulfur, boron, iron, and manganese were present as 4.84-5.41 $\mu g\ kg^{-1}$, 0.416 to 2.12 $\mu g\ kg^{-1}$, 0.63-16.74 $\mu g\ kg^{-1}$ and 3.30 to 5.41 $\mu g\ kg^{-1}$ soil respectively.
- The recovery of DEP in MS broth was 94.8 to 95.3% and in soil 91.6-91.96% while the recovery of DEHP in MS broth 90.93 to 91.33% and in soil microcosm 93.4 to 93.76%, respectively.
- Enrichment technique was used for the isolation of PAEs degrading bacteria from the three soil samples collected from different sites at Centre for Cultivated Protection Technology (CPCT) IARI, New Delhi, India.
- A total ninety bacterial isolates were selected on visual basis which was further screened for morphological characterization like pigmentation, colour, margin, shape, and size.
- Most of the colonies were whitish in colour, however, variation in white colour was observed from dull, creamy white to off-white while two colonies were shiny with blackish shade. Margins of colonies varied from circular, irregular to punctiform with entire, curled and undulated margin. Some of colonies were pigmented. The margins of colonies were either flat or raised.
- 18 selected isolates were found to be rod-shaped except DEP B2 which was cocci.
- The nine DEP degrading bacterial isolates were further enzymatically characterized where all the nine isolates were found to produce cytochrome oxidase, degrade H_2O_2 by producing enzyme catalase and able to hydrolyze esters with the help of esterase enzyme.

- The nine bacterial isolates for DEHP degradation were also characterized enzymatically and all nine were found to produce cytochrome oxidase, catalase, and esterase but variation was again observed for urea and starch hydrolysis.
- When nine DEP and nine DEHP bacterial isolates were grown on different concentration from 100 ppm to 700 ppm of respective compound in mineral salt medium broth, all isolates showed an increase in protein concentration with simultaneous increase in DEP and DEHP concentration till 300 ppm.
- The nine isolates for DEP degradation also showed variation in protein and almost five isolates DEP A1, DEP A2, DEP B2, DEP B3, and DEP C3 were able to grow at 300 ppm DEP while other four isolates showed good growth at 100 ppm DEP, moderate at 300 ppm, low at 500 ppm and poor at 700 ppm.
- The final eleven selected bacterial isolates were further studied for molecular characterization. The phylogenetic relationships of the isolates as inferred from comparison of partial sequences (approx 1500bp) of the 16S rRNA genes showed that these isolates fell into two major lineages of domain bacteria; the γ -Proteobacteria and β -Proteobacteria.
- The ten isolates of γ -Proteobacteria, matched with sequences of *Pseudomonas aeruginosa* (DEHPA1, DEHPB1, and DEHPC3), *Pseudomonas sp.* (DEHPA2, DEHPB2, and DEHPC2), *Pseudomonas plecoglossicida* (DEPB3) and *Enterobacter sp.* (DEPA1 and DEPC1).
- The one isolate of β -Proteobacteria, matched with sequences of *Achromobacter* (DEPA3).
- All the identified strains for DEP and DHEP were found compatible with each other as no antagonistic activity was observed on plates.
- The three isolates viz. *Pseudomonas sp.* DEHPA2, *Pseudomonas sp.* DEHPB2 and *Pseudomonas sp.* DEHPC2 were inoculated individually and also as mixed inoculum in equal ratio (1:1:1) of three together as consortium in MS broth containing 300 mg L⁻¹ DEHP as sole carbon source.
- The most efficient degradation was observed when the three bacteria were inoculated as consortium and from initial 285 ppm DEHP a rapid decline was

observed as 49.1 ppm, 32.4 ppm, and 11 ppm after 10, 20 and 30 days of incubation respectively. The DEHP % degradation was observed 79.8 - 87.8% after 10 days, 87.1- 91.3% after 20 days and 88.7 to 96.6% after 30 days of incubation.

- The results highlighted the role of selected bacterial isolates for degradation of DEHP in MS broth. The maximum degradation within 30 days was observed with mixed culture as compared to individual bacterial isolates.
- After MS broth study, the three selected bacteria *Pseudomonas sp.* DEHPA2, *Pseudomonas sp.* DEHPB2 and *Pseudomonas sp.* DEHPC2 were inoculated in soil for degradation of DEHP. All the bacterial isolates were inoculated individually as well as a consortium (1:1:1) in 50 gm soil containing 300 ppm DEHP
- Based on degradation pattern, the DEHP percentage degradation was also observed. The variation in percentage DEHP degradation among the bacterial isolates varied from 72.2 to 88.5% from 10 days to 30 days of incubation but a good DEHP percentage degradation was observed with mixed inoculum at 10, 20 and 30 days as compared to individual bacterial isolates.
- The mixed inoculum (MI) was found to be most efficient in DEHP degradation where the decline in DEHP was observed from initial 276 ppm DEHP to 67.8 ppm, 54.4 ppm and 21 ppm at 10, 20 and 30 days of incubation.
- Three bacterial isolates viz. *Achromobacter xylosoxidans* strain DEPA3, *Pseudomonas plecoglossicida* strain DEPB3 and *Enterobacter cloacae* strain DEPC1 were selected for the degradation of DEP in MS broth with 300 mg L⁻¹ DEP as sole carbon source. Each bacterial culture was inoculated individually and as a mixed culture (1:1:1) in MS broth and kept for incubation for 30 days. An un-inoculated broth was kept as control.
- A rapid degradation of DEP was observed with mixed inoculum showing 9.5 ppm DEP after 30 days which was the maximum and among the bacteria *Enterobacter cloacae* strain DEPC1 was found superior over other two strains *Achromobacter xylosoxidans* strain DEPA3, *Pseudomonas plecoglossicida* strain DEPB3 for DEP degradation in MS broth. The

reduction of pH in the broth was observed in all the inoculated flasks as compared to un-inoculated flasks and the range of pH reduction was observed as 6.1 -6.3.

- The percentage degradation increased with time where after 30 days mixed inoculum showed 96.14% degradation followed by *Pseudomonas plecoglossicida* strain DEPB3 (85.76%) while *Enterobacter cloacae* strain DEPC1 and *Achromobacter xylosoxidans* strain DEPA3 showed marginal differences with 84.70 and 84.91 % respectively
- The three selected bacteria namely viz. *Achromobacter* strain DEPA3, *Pseudomonas plecoglossicida* strain DEPB3 and *Enterobacter cloacae* strain DEPC1 were further evaluated for the degradation of DEP in soil microcosm. The bacterial inoculation was done individually and also as mixed inoculum (MI) in 50 gm of soil having 300 ppm DEP at start of experiment. An un-inoculated control was also kept throughout the experiment.
- The percentage degradation of DEP in soil microcosm was also estimated at different time intervals and it was observed that the bacterial culture *Achromobacter xylosoxidans* strain DEPA3 showed 66.2 to 81.6% DEP degradation within 30 days while *Pseudomonas plecoglossicida* strain DEPB3 resulted in 67.31 to 82.24% DEP degradation and *Enterobacter cloacae* strain DEPC1 with 65.85 to 81.74% DEP degradation in soil microcosm from 10 days to 30 days of incubation.
- The mixed inoculum comprising of all these three bacterial culture (1:1:1) resulted as the most efficient degraders in 30 days where the range for percentage DEP degradation was observed as 75.43 - 92.39%. These results clearly indicate the role of mixed inoculums or a consortium for rapid degradation of DEP in soil microcosm.
- The results of this study indicate that ability to degrade DEP and DEHP is widely distributed among phylogenetically diverse group of bacteria in irrigated agricultural soils. The enriched mixed inoculum has proved to be better scavenger of DEP and DEHP in soil as compared to individual isolates. The potential isolates obtained in the present study can be used for developing consortium which can be tested further at larger scale to strengthen the findings of the study.

Biodegradation of phthalate esters using efficient microorganisms

Abstract

The present study was carried out for isolation, identification and characterization of DEP and DEHP degrading bacteria from Centre for Cultivated Protection Technology (CPCT) IARI, New Delhi. Phthalate esters are categorized according to their molecular weight. DEHP is classified as higher molecular weight esters while DEP as low molecular weight. Both the PAEs are well-known xenobiotics and recalcitrant nature. Phthalate esters and their metabolites are known to exhibit carcinogenic action and also found to be endocrine disruptors and produce adverse reproductive, neurological and immune effect in both human and wildlife.

In solving the serious problems of pollution caused by phthalate esters, it is important to assess the potential of bacterial strains indigenous to sites, contaminated with phthalate esters for their ability to degrade DEP and DEHP. The eleven bacterial isolates were identified through 16S rDNA sequencing, which grew well at 500 ppm of DEP and DEHP as sole carbon source in minimal salt medium. However, reduction in their growth was observed at 700 ppm both DEP and DEHP. Out of eleven isolates, ten isolates belonged to class γ *Proteobacteria*, matched with sequences of *Pseudomonas* and *Enterobacter*. One isolate *Achromobacter* belonged to β - *Proteobacteria*. DEP degradation by *Achromobacter xylosoxidans* strain DEPA3 is reported for the first time in the present study and these might represent new DEP-degrading taxa.

HPLC studies revealed decrease in the residual DEP and DEHP and formation of intermediates such as Phthalic acid by selected isolates. The three isolates viz *Pseudomonas* sp. DEHPA2, *Pseudomonas* sp. DEHPB2 and *Pseudomonas* sp. DEHPC2 were inoculated individually and also as consortium in MS broth containing 300 mg L⁻¹ DEHP as sole carbon source.

The most efficient degradation was observed when the three bacteria were inoculated as consortium. The DEHP percentage degradation was observed 79.8 - 87.8% after 10 days, 87.1- 91.3% after 20 days and 88.7 to 96.6% after 30 days of incubation in MS broth. Also, study in soil microcosm and the variation in percentage DEHP degradation among the bacterial isolates from 72.2 to 88.5% from 10 days to 30 days of incubation but a good DEHP percentage degradation was

observed with consortium of three bacteria. Three bacterial isolates namely viz. *Achromobacter xylosoxidans* strain DEPA3, *Pseudomonas plecoglossicida* strain DEPB3 and *Enterobacter cloacae* strain DEPC1 were selected for the degradation of DEP in MS broth and soil microcosm in different time interval by individuals and consortium of three bacteria. In MS broth (84.91 - 96.14%) observed more DEP degradation than soil microcosm (81.16-92.39%). The potential isolates obtained in the present study can be used for developing consortium which can be tested further at larger scale to strengthen the findings of the study.

दक्ष सूक्ष्मजीवों द्वारा थैलेट एस्टरस का जैव अवक्रमण

सारांश

वर्तमान अध्ययन, सेंटर फॉर कल्टीवाटेड प्रोटेक्शन टेक्नोलॉजी भारतीय (सीपीसीटी) संस्थान अनुसंधान कृषि, नई दिल्ली से डीईपी और डीईएचपी अवक्रमित जीवाणु के पृथक्करण, अभिज्ञान और लक्षण विवरण के लिए किया गया था। थैलेट एस्टर को उनके आणविक भार के अनुसार वर्गीकृत किया जाता है I डीईएचपी को उच्च आणविक भार एस्टर के रूप में वर्गीकृत किया जाता है जबकि डीईपी कम आणविक वजन के रूप में वर्गीकृत किया जाता है। पीएई दोनों प्रसिद्ध ज़ीनोबिओटिक्स और दुर्दम्य प्रकृति हैं। थैलेट एस्टर और उनके मेटाबोलाइट्स कैंसरजन्य क्रिया को प्रदर्शित करने के लिए जाना जाता है और यह अंतःस्त्रावी विघटनकर्ता भी पाया जाता है और मानव और वन्यजीवन दोनों में प्रतिकूल प्रजनन, तंत्रिका विज्ञान और प्रतिरक्षा प्रभाव उत्पन्न करता है। थैलेटएस्टर द्वारा प्रदूषण की गंभीर समस्याओं को हल करने में, थैलेट एस्टर से दूषित साइटों के लिए स्वदेशी जीवाणु उपभेदों की, उनकी डीएचपी और डीईएचपी को अपनाने की क्षमता के लिए आकलन करना महत्वपूर्ण है। ग्यारह जीवाणु पृथक् 16 एस आरडीएनए अनुक्रमण के माध्यम से पहचाने गए थे, जो न्यूनतम नमक माध्यम स्रोत डीईपी और डीईएचपी के 500 पीपीएम एकमात्र कार्बन पर अच्छी तरह से बढ़े। हालांकि, डीईपी और डीईएचपी दोनों में 700 पीपीएम पर उनकी वृद्धि में कमी देखी गई। ग्यारह अलगाव में से, दस पृथक् वर्ग γ प्रोटेबोबैक्टेरिया से संबंधित थे, जो स्यूडोमोनास और जीवाणुओं को एमएस ब्रॉथ में जिसमें डीएचएचपी 300 मिली ग्राम प्रति लीटर को एकमात्र कार्बन स्रोत एंटरोबैक्टर के अनुक्रमों से मेल खाते थे। एक अलग जीवाणु एक्रोमोबैक्टर, बीटा-प्रोटेबोबैक्टीरिया से संबंधित था। वर्तमान अध्ययन में पहली बार आक्रोमोबैक्टर जयलोलोसोक्सिडेंस डीईपीए 3 अवक्रमण रिपोर्ट किया गया है और ये नए डीईपी-अपघटन कर का प्रतिनिधित्व कर सकते हैं। एचपीएलसी अध्ययनों ने अवशिष्ट डीईपी और डीईएचपी में कमी और चयनित जीवाणु द्वारा थैलेट एसिड जैसे मध्यवर्ती के गठन का खुलासा किया। स्यूडोमोनास जाति डीएचएचपीए 2, स्यूडोमोनास जाति डीएचएचपीबी 2 और स्यूडोमोनास जाति डीएचएचपीसी 2 तीन अलग के रूप में शामिल किया गया था में व्यक्तिगत और संघ रूप में अध्ययन किया गया।

जब तीन बैक्टीरिया को संघ के रूप में लगाया गया था तब सबसे कुशल अपघटन पाया गया। डीएचएचपी अपघटन का एमएस ब्रॉथ में विघटन प्रतिशत समय के साथ अलग-अलग पाया गया। 10 दिनों के बाद 79.8 - 87.8%, 20 दिनों के बाद 87.1- 91.3% और 30 दिनों के बाद 88.7 से 96.6% प्राप्त हुआ। इसी तरह मिट्टी के सूक्ष्मदर्शी में भी अध्ययन किया गया और जीवाणुओं के बीच प्रतिशत

डीएचएचपी अपघटन में भिन्नता देखी गई तथा 10 से 30 दिनों में 72.2% से 88.5% की प्राप्ति हुई लेकिन तीन बैक्टीरिया के संघ के साथ एक अच्छा प्रतिशत डीईएचपी अपघटन प्राप्त हुआ। तीन बैक्टीरिया द्वारा जिनका नाम एक्रोमोबैक्टर जयलोलोसोक्सिडेंस स्ट्रेन डीपीए 3, स्यूडोमोनास प्लेकोग्लोसिसिदा स्ट्रेन डीईपीबी 3 और एंटरोबैक्टर क्लॉएसी स्ट्रेन डीईपीसी 1 और उनके संघटन को डीईपी के क्षरण के लिए एमएस ब्रॉथ और मिट्टी माइक्रोक्रोस में अलग-अलग समय अंतराल में चुना गया था। एमएस ब्रॉथ (84.9 ± 1 - 96.14%) में मिट्टी माइक्रोक्रोस (81.16 - 92.39%) की तुलना में अधिक डीईपी क्षरण पाया गया। वर्तमान अध्ययन में प्राप्त बैक्टीरिया संघ के विकास के लिए संभावित उपयोग किया जा सकता है जिसे अध्ययन के निष्कर्षों को मजबूत करने के लिए बड़े स्तर पर परीक्षण किया जा सकता है।

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